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EVALUATION OF IMMOBILIZED CELL FERMENTATION FOR CANOLA SAUCE PRODUCTION

BY

CLAUDIA MARTENS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

FOOD PROCESS ENGINEERING

DEPARTMENT OF FOOD SCIENCE AND NUTRITION

EDMONTON, ALBERTA

Fall, 1994

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(SIGNED) Claudia Martens

PERMANENT ADDRESS: c/o B. Immler
Wellenburgerstraße 26
86199 Augsburg, GERMANY

Date: July 20, 1994

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **EVALUATION OF IMMOBILIZED CELL FERMENTATION FOR CANOLA SAUCE PRODUCTION** submitted by **CLAUDIA MARTENS** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in **FOOD PROCESS ENGINEERING**.

Dr. R. R. Segado

Dr. B. Ooraikul

Dr. F. J. A. Merchant

Dr. J. S. Sim

Date: July 20, 1994

ABSTRACT

A soy-like canola sauce was produced in 12 days. The process consisted of a traditional koji step, followed by a 3-day hydrolysis with the enzymes produced in the koji. The solid-free extract was then subjected to ethanol fermentation by *Zygosaccharomyces rouxii* immobilized in two different porous support materials: Labpor, made of high density polyethylene, and Biofix C2, a material derived from kaolinite. The same support particles containing yeast cells were used for 3 consecutive batches. Once the alcohol content had reached 1.5% (w/v), the broth was drained and replaced by a fresh medium. The fermentation time for one batch varied between 5-8 days. The productivity of the immobilized cells slowed down considerably after about 300 h of fermentation, probably due to the presence of an unusually high amount of lactic acid in the feed medium and/or fouling of the support particles.

The resulting canola sauce contained 1.51% (w/v) ethanol, 0.362% (w/v) glycerol, 1.79% (w/v) total soluble nitrogen (TSN), 0.775% (w/v) amino nitrogen (AN), 0.113% (w/v) glucose, 25.2 (meq NaOH/100 mL sauce) total acidity and a pH of 4.52. The sauce had colour similar to that of Kikkoman soy sauce, but it had a lower glycerol and lower residual glucose content than the commercial sauce.

Biofix was found to be a better support material due to its higher cell load in g (wet weight) per kg support material: 27.1 g/kg compared to only 15.6 g/kg for Labpor.

Canola sauce and soy sauce were also produced using the traditional method, which consisted of a koji step followed by a 4-month

moromi fermentation. The resulting sauces were similar in composition except that the traditional soy sauce had a higher AN, TSN and residual glucose content. The traditional soy sauce contained 0.681% (w/v) AN, 1.46% (w/v) TSN, and 0.824% (w/v) glucose, whereas the traditional canola sauce contained 0.530% (w/v) AN, 1.20% (w/v) TSN and 0.447% (w/v) residual glucose.

The canola sauce produced with immobilized cells was darker in color, had a significantly lower sugar content and a higher glycerol content than the traditional canola sauce.

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CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

Historical aspects of soy sauce

Soy sauce (or Shoyu in Japanese) is a liquid food condiment prepared by fermentation of soybeans or soybean meal and wheat. It is the most popular sauce in oriental countries and is characterized by its distinctive aroma and flavour. Soy sauce has also gained acceptance in other parts of the world, such as Europe, United States and Canada.

Soy sauce was derived from 'chiang', a chinese, semi-solid, tasty, fermented mash product made from millet and meat or fish, which originated in China. The first record on chiang was found in a book published around 1100 BC in the Chou dynasty (1122 BC to 249 BC) (Pao, 1982a, b). The production process has always involved the growth of mould on a substrate in order to produce enzymes which break down this substrate. Chinese people started using the enzymatic action of mould in food manufacturing as early as 3000 years ago. The technology was developed well before the biochemical processes involved in microbial degradation of raw materials by microorganisms were known (Yong and Wood, 1974).

The meat and fish in the chiang were gradually replaced by soybeans, and later, cereals such as wheat, barley and rice came to be used instead of millet. The liquid products which belong to the category of soy sauce did not appear in the literature until the Han dynasty (about 25-

220 AD) (Fukushima, 1989). The introduction and development of soy sauce into Japan in the sixth century was believed to be associated with Buddhism, in which meat was abstained from the diet (Fukushima, 1979).

Types of soy sauce

Five types of soy sauce are classified by the Japanese Agricultural Standard (JAS): Koikuchi-shoyu, Usukushi-shoyu, Tamari-shoyu, Saishikomi-shoyu and Shiro-shoyu. The typical composition of these varieties of soy sauce is shown in Table 1.1. Each type is classified into three grades, namely special, upper and standard. Koikuchi shoyu accounts for more than 85% of the total soy sauce production in Japan and is considered as the representative Japanese soy sauce (Fukushima, 1985).

The grade of the sauce is determined by organoleptic evaluation, total nitrogen content, soluble solids without NaCl content, and alcohol content. Special grade is assigned to high quality sauce made only by the action of microorganisms. The use of chemical and/or enzymatic hydrolyzates for special grade sauce is not permitted (Fukushima, 1989). In 1981, about 60% of Japanese soy sauce was special grade (Yokotsuka, 1986a).

Soy sauce can be produced in 3 different ways: by fermentation, by a semi-chemical process and by a chemical process. However, a sauce produced by the chemical process is not recognized as soy sauce by the Japanese Government (Fukushima, 1985).

For the fermented sauce, the raw material is slowly hydrolyzed by enzymes produced by the koji mould, followed by a lactic acid and alcohol

fermentation. For the chemical sauce process, which is presented in Fig. 1.1, the proteins and carbohydrates are hydrolyzed very quickly by hydrochloric acid at temperature >80°C for 12-16 hours (Fukushima, 1985). It is a very cheap and quick process, but during the hydrolysis various secondary reactions occur which produce undesirable compounds, such as dark humins, furfural, dimethyl sulfide, hydrogen sulfide, levulinic acid, formic acid, and other compounds, which are not present in fermented sauce (Fukushima, 1985). Furthermore, tryptophan, one of the nutritionally important amino acids, is destroyed almost completely (Fukushima, 1985). Levulinic acid, which does not exist naturally, can be used as an indicator to determine if a fermented sauce has been extended with chemical sauce.

Another process was developed involving a milder chemical hydrolysis followed by fermentation with lactic acid bacteria and yeast. The resulting product is a semi-chemical sauce, but it does not have the typical flavour of fermented sauce and a small amount of the characteristic furfural and sulfide-like odors are still unavoidable (Yong and Wood, 1974).

Traditional production process for soy sauce

The traditional manufacturing process of fermented soy sauce consists of three major steps: koji making, moromi fermentation, and refining. Fig. 1.2 shows the production steps for the manufacture of traditional soy sauce.

For koji making, a 1:1 mixture of soybeans and wheat is usually used. Wheat grains are roasted until they turn slightly brown and are then coarsely ground. Before 1910, soybeans were soaked and then steamed in a large steam cooker under atmospheric pressure for about one hour. By this

method, the yield of soy sauce only ranged from 60 to 63% (Fukushima, 1989). It was later discovered that the higher the cooking pressure and temperature, the greater the yield, whereas the longer the time the lower the yield (Fukushima, 1985). Since then, the pretreatment for soybeans was done at high temperature in the shortest time possible, with rapid cooling following the heat treatment (Fukushima, 1989).

The mixture of soybeans and wheat is then inoculated with koji starter culture, usually *Aspergillus sojae* and/or *Aspergillus oryzae*. The amount of mould added is usually 0.1-0.2% of the raw material weight (Yokotsuka, 1985b). This mixture is spread on shallow perforated vats or stainless steel plates and kept for about 72 h in a moisture controlled environment with temperatures ranging from 28-30°C and humidity close to saturation. During mould growth, it is important to control the temperature of the koji. This is usually done by stirring the cultured mixture about 20 h and 25 h after inoculation (Fukushima, 1989). If koji is stirred too late, its temperature can become too high, which might lead to a decrease in enzyme production and sometimes to the death of the mould (Fukushima, 1989). After 3 days the mould covers the wheat/soybean mixture. It has a yellowish-green appearance as a result of sporulation, and contains the necessary enzymes to hydrolyse proteins and carbohydrates during the moromi fermentation (Yokotsuka, 1986a, b).

The mature koji is mixed with 120 to 130% (v) of water containing 22 to 23% salt in a fermentation tank (Yokotsuka, 1985a). The resulting mixture is called moromi or moromi mash. It is then held for at least 6 months under appropriate temperature controls with occasional brief aeration to mix the contents and to stimulate microbial growth. During moromi fermentation, the

enzymes from koji hydrolyze proteins to amino acids and small peptides. Starch is converted to simple sugars, which are fermented primarily to lactic acid, alcohol and carbon dioxide. The high salt concentration limits the growth of microorganisms to a few desirable osmophilic species. The final process of soy sauce making is refining. The aged moromi is put into a fine cloth and pressed in a hydraulic press until the water content of the residue is less than 25% (Fukushima, 1985). The raw sauce is then heated to 70-80°C for 30 minutes in a plate heat exchanger, filtered and bottled.

Microbiology and biochemistry of koji and moromi

Koji

The use of koji is analogous to the use of malt in the Western nations. Its use as a starter is not exclusive to the shoyu industry, it is also used in the brewing of the Japanese rice-wine (sake) and fermentation of miso (a sort of porridge or mush made by mixing mashed steamed soybeans, salt and koji, and by allowing the mixture to ferment) (Yong and Wood, 1974).

Koji making is a very unique technology to use microorganisms cultured on a solid medium of cereals as an enzyme source. It contains not only macerating, amylolytic and proteolytic enzymes in large quantities with a well-balanced ratio, but also their proteolytic enzymes are composed of a very unique system of proteinases and peptidases (Fukushima, 1985). Table 1.2 presents the specific reactions of koji enzymes. The actions of these proteolytic enzymes are considered the most important process in the production of soy sauce. Fukushima (1985) stated that 90-92% of the proteins contained in the raw materials for soy sauce production are

hydrolyzed into the liquid phase as free amino acids by the proteolytic enzymes.

The selection of mould strains used for food fermentation is based on the following criteria: flavor and color of the final product; good spore forming ability, which is necessary to prepare the seed starter; strong and rapid growth; high enzymatic activity, especially of proteolytic and macerating enzymes; low consumption of starch during growth; genetic stability; length of stalk (short-stalk strains are most suitable for mechanical koji cultivation); and no toxin production (Yokotsuka, 1985b). Maximum proteolytic activity is obtained from a koji containing equal parts of wheat and soybeans (Yamamoto, 1957).

Yokotsuka (1972) and Yong and Wood (1977) showed that most of the important enzymes reach their maximum level after about 50 h of incubation of the koji. If the koji ages excessively, with considerable sporulation, the resulting sauce will have a mouldy off-flavour and an excessive ammonia content. Yong and Wood (1977) reported that as the koji aged beyond the 72 h mark, profuse sporulation developed and the mild, slightly musty smell of the young koji was replaced by a harsh, mouldy smell.

Mold strains with high production of both amylolytic and proteolytic enzymes are used (*A. oryzae* and/or *A. sojae*). It is known that the proteinases of those two strains are composed of seven kinds of proteinases with four different optimum pH levels. The proteinases are all endo-type enzymes which do not possess any amino- or carboxy-peptidase actions. Accordingly, they hydrolyze proteins only into peptides. Free

amino acids are not liberated substantially by these proteinases (Fukushima, 1985).

Koji also contains various kinds of exo-type peptidases, which liberate the amino acid from the carboxy- or amino-terminal of peptides successively. So far, four kinds of carboxypeptidases and seven kinds of aminopeptidases have been isolated from koji (Fukushima, 1985).

Moromi

The initial pH of moromi mash is around 6.5-7.0. The types of microorganisms able to grow in the mash are limited by the high salt concentration (18%). Non salt-tolerant microorganisms such as wild yeasts, *Micrococcus* and *Bacillus* are destroyed or stop growing at the beginning of the moromi fermentation, and only salt-tolerant yeasts and salt-tolerant lactic acid bacteria remain active (Yokotsuka, 1985b). The higher the salt concentration in the mash, the lower is the water activity (a_w). The a_w value is related to the amount of water available for microbial growth. The major lactic acid bacterium in shoyu mash was shown to be *Pediococcus halophilus*. It can grow in 24% salt solution in the temperature range of 20-42°C. The initial pH-value of shoyu mash rapidly decreases due to enzymatic degradation of proteins and lactic acid fermentation (Yokotsuka, 1985a). Only when its pH reaches a value of about 5.0, *Saccharomyces rouxii* begins to grow and, as a result, a vigorous alcoholic fermentation occurs.

The majority of yeasts can grow with a minimum a_w level of 0.88. *S. rouxii*, however, adapts itself to growth in a medium of diminished water activity. It synthesizes polyols, mainly glycerol and a small amount of

arabitol, which serve not only as osmoregulators but protect enzymes from damage due to low a_w (Board, 1983). The cell membranes of the osmotolerant yeast strains retain the polyols within the cell, whereas those of non-osmotolerant yeasts are leaky and much of the osmoregulators produced in response to a diminished a_w is lost to the surrounding medium (Board, 1983).

The dominant strain of yeast found in shoyu mash is *S. rouxii*, but *Torulopsis* yeasts such as *T. versatilis* and *T. etchellsii* are also found (Yokotsuka, 1985a). These yeasts can grow in a pH 3-7 in salt free medium, but this range is narrowed to pH 4-5 in 18% salt solution (Ohnishi, 1963). Fig 1.3 shows the changes in microflora in shoyu mash fermentation.

Proteins and carbohydrates are degraded by proteases, peptidases, amylase, pectinase and phosphatase (Yokotsuka, 1986b). The proteins are degraded into lower peptides, free amino acids and ammonia. The carbohydrates are hydrolyzed to hexoses and pentoses, and are metabolized partly into about 1% lactic acid and other organic acids by lactic acid bacteria, and partly into 2-3% of ethanol and other minor flavorous compounds by yeasts. About 2-4% of glucose and trace amounts of xylose usually remain in the final mash (Yokotsuka, 1985b). The enzymatic degradation stops after 2 or 3 months from the beginning of the moromi fermentation (Yokotsuka, 1985a). After the fermentation is complete, an additional 3-4 months are necessary to finish the moromi aging, during which the so-called browning reactions such as Strecker degradations and other reactions occur (Yokotsuka 1985b).

In the moromi fermentation, microbial antagonism has been observed between osmophilic lactic acid bacteria and yeasts. The inhibitory effect of

osmophilic lactic acid bacteria on the growth of osmophilic soy sauce yeast is considered to be due to metabolites produced by *P. halophilus*. The primary inhibitor contained in the metabolite is acetic acid, although lactic acid is also slightly inhibitory (Noda *et al.*, 1980).

Chemical composition of soy sauce

Nearly 300 flavour components have been identified in Japanese fermented soy sauce (Nunomura, 1992). All processes in the soy sauce production are related to the formation of flavour compounds, namely, heat treatment of raw materials, koji culturing by moulds, lactic acid fermentation, yeast fermentation in moromi mash, and pasteurization of the sauce. Among these processes, yeast fermentation contributes the most to flavour development (Nunomura, 1992).

In general, good-quality regular soy sauce (koikuchi-shoyu) has a pH of 4.7-4.8 and contains 1.5-1.65% (w/v) total nitrogen (simple peptides: 45%, amino acids: 45%, and ammonium: 10%), 2-5% (w/v) reducing sugars (about 80% glucose), 1-2% (w/v) organic acids (about 70% lactic acid), 1.0-1.5% (w/v) polyalcohols (80-90% glycerol), 2.0-2.5% (v/v) ethanol, and 16-18% (w/v) sodium chloride (Fukushima, 1985). Table 1.3 presents typical analytical data for sugars, organic acids and alcohols in koikuchi-shoyu.

Acceleration of the fermentation process

The increase in protein digestability of soy sauce due to recent improvements in the process of soybean cooking and koji cultivation has helped to reduce the fermentation period from the 1-3 years required in the past to less than one year (Yokotsuka 1986b). Completing the fermentation

process within 6 months without excessively lowering the quality of the final product can be accomplished by keeping the moromi mash at a temperature of about 30°C. However, using higher temperatures would reduce the organoleptic qualities of the resulting product (Yokotsuka 1986b).

It has been found by Takamatsu *et al.* (1980) that the process could be shortened considerably by mixing the shoyu koji with a sodium chloride solution (1-12%) and agitating it vigorously. Part of the raw material can be hydrolyzed in this manner. The relationship between salt concentration and temperature to avoid microbial contamination in the enzymatic degradation of koji is shown in fig. 1.4. The shaded area presents the safety zone for enzymatic digestion of shoyu koji. It has been found that the protein digestability and amino acid content in zone A are better than in zone B. After this hydrolysis, it still takes 2-3 months to complete the moromi fermentation of the mash at 20-30°C. However, the liquid hydrolyzate can be separated from the cake and the fermentation can be finished within a few days using immobilized lactic acid bacteria and yeast cells (Yokotsuka, 1986b).

Alternative raw materials

Several attempts have been made to make 'soy sauce' using raw materials other than soybeans and wheat, but most of them were unsuccessful commercially.

Church (1923) replaced soybeans by peanut press cake, but the resulting sauce had an evident peanut taste. During the second world war, the Japanese were so desperate for raw materials, that they even tried to use garbage as a substitute (Tsukahara, 1948). Oda *et al.* (1949) prepared

a substitute 'soy sauce' using acorns and wheat with an *Aspergillus* culture that produces tannase. Baens-Arcega (1966) successfully used a 50:50 mixture of copra meal and soybeans as a cheap source of proteinaceous material for soy sauce production.

Ooraikul *et al.* (1980) demonstrated that it is possible to replace soybean meal with rapeseed meal for the production of 'soy sauce'.

Canola meal

Canola meal is the residue left after the oil is extracted from the canola seed. In developing canola in the mid seventies, researchers genetically altered the widely grown *Brassica napus* and *Brassica campestris* species of oilseed plants generally known as rapeseed (Clandinin, 1986). Canadian plant breeders developed low erucic acid, low glucosinolate (double low) cultivars of the oilseed. All of Canada's rapeseed crop is now of this type and generally referred to as "canola" to distinguish it from cultivars with low erucic acid and high glucosinolate, and cultivars with high erucic acid and high glucosinolate grown in other parts of the world (Clandinin, 1986).

Canola oil supplies 46% of the vegetable oil in the diet of Canadians while canola meal is used extensively by the feed industry as a source of high quality protein in rations for livestock and poultry (Clandinin, 1981). Table 1.4 shows the proximate composition and the amino acid composition in protein percent of canola meal and soy bean meal. The protein content of canola meal is lower than that of soy meal. However, the amino acid content compares favourably with that of soy meal. The crude fibre content of canola meal is higher than that of soybean meal. While efforts are being

made to reduce the fibre content of canola meal by dehulling or air classification and by breeding cultivars of canola which have thin hulls, a solution to the high crude fibre content has not yet been found (Clandinin, 1986).

Reduction of the total fermentation time of canola sauce.

The canola sauce developed by Ooraikul *et al.* (1980) was made by fermenting canola meal and wheat, on which *A. oryzae* and/or *A. soyae* was grown for one year. The resulting product was found to be comparable in its acceptability to a commercial soy sauce. To reduce the long fermentation time Ooraikul *et al.* (1980) also used a semi-chemical process adapted from Hesseltine and Wang (1972). The fermentation could be shortened to about one month, but the high cost of acid resistant equipment and food grade chemicals as well as the formation of undesirable compounds, made this method unattractive.

To overcome these problems, Ma and Ooraikul (1986) replaced the acid hydrolysis by a milder enzymatic hydrolysis of canola meal using the enzyme Alcalase 0.6L (a protease). Canola meal was prehydrolyzed for 2 h with Alcalase 0.6L before mixing it with wheat and *Aspergillus* cultures for koji. Once koji was mature, moromi mash was prepared as usual, but was only allowed to ferment naturally for 5 weeks. The resulting canola sauce was rated inferior to Kikkoman shoyu. Concentrations of amino acids, especially glutamic and aspartic acids, were lower than in the commercial soy sauce. The concentrations of organic acids were comparable, except for lactic and propionic acid, which were considerably lower in the canola

sauce due to inadequate lactic acid fermentation in the moromi stage. Further modifications were necessary to improve this sauce.

Coleman and Ooraikul (1989) improved the sauce by inoculating the canola mash before a 4-week moromi fermentation with pure cultures of *P. halophilus*, *S. rouxii* and *T. versatilis*. The chemical characteristics of the resulting canola sauce were similar to that of Kikkoman shoyu. But an excessive amount of lactic acid and the absence of some characteristic components gave the canola sauce a distinct "sharp taste" and "raw flavour".

To shorten the total production time down to a few days, Ningsanond (1991) used a combination of industrial enzymes to replace the koji step, followed by a fermentation using immobilized cells to replace the moromi step. The total production time of the canola sauce was shortened to 3-4 days. The enzymatic hydrolysis used by Ningsanond (1991) is shown in Fig. 1.5.

In the traditional fermentation, the enzymes produced during the koji step are still active during the moromi step, hydrolyzing canola meal and wheat . In order to carry out the moromi fermentation with immobilized cells, it is important that the feed medium be liquid, unlike the traditional moromi fermentation in which the medium is semi-solid and still contains large molecules and undigestable material (fibre). By using industrial enzymes and then separating the dissolved material from the undissolved material, a feed medium was obtained that was suitable for immobilized cell fermentation (Ningsanond, 1991).

Nevertheless, the sauce produced by Ningsanond (1991) had generally significantly lower sensory scores than the Kikkoman soy sauce.

This was partially due to the low amino nitrogen/total soluble nitrogen (AN/TSN) ratio, indicating a low degree of amino acid hydrolysis.

Immobilized cells

For the industrial exploitation of microorganisms, immobilized cells offer certain advantages over free cells, such as the possibility of reusing the cells and simplified downstream processing. Use of immobilized cells also permits higher biocatalyst loads and facilitates continuous operation, thus reducing equipment costs. However, due to diffusional limitations of substrates and metabolites in the immobilization media, fermentation rates may be lower when using immobilized cells than with free cells.

The first industrial application of immobilization was in 1969. The Japanese firm of Tanabe Seiyaku immobilized an L-aminoacylase from *A. oryzae* by ionic binding on DEAE-Sephadex to be used in the production of L-amino acids (Chibata and Tosa, 1981). The industrial production of high-fructose corn syrup using immobilized glucose isomerase followed shortly thereafter (Witter, 1992).

Cell immobilization can be achieved by binding or physical retention. Of the immobilization methods presented in table 1.5, entrapment or weak adsorption are the most commonly used (Witter, 1992). Because some reagents used for covalent binding and cross-linking are toxic to the cell (e.g. diisocyanate or cyanogen bromide), these methods can only be used if dead, immobilized cells are satisfactory.

In the process of entrapment, whole cells are enclosed in a gel-like matrix. This is often the method of choice, but these gel beads present certain disadvantages. The lack of mechanical strength limits their use in

large column reactors, while cell growth and gas production can cause rupture of the gel during the fermentation process. Furthermore, application of alginate gels is limited by their sensitivity to chelating agents, such as polyphosphates and citric acid, which disrupt the gel structure by binding calcium.

A gel also presents the disadvantage of diffusional limitations, and, in a spherical matrix, for example, the cells near the centre may not participate in the biological process (Castillo *et al.*, 1991; Westrin and Axelsson, 1991). Rao *et al.* (1986) tried to overcome the diffusional limitations of agar beds by using yeast cells entrapped in open-pore gel beads, which were obtained by selective leaching of calcium alginate from an agar-calcium alginate composite matrix.

An alternative method for immobilization is the adsorption of cells on a porous or non porous material. This method is probably the least expensive and mildest method of immobilization. It involves weak interacting forces such as hydrogen bonds, hydrophobic interactions, and Van der Waal forces (Witter, 1992). Cells tend to create their own biospecific interactions with appropriate surfaces, and even cells normally considered as preferring suspension culture will invade some open structures and lay down an adhesive film (Rosevear *et al.*, 1987). Porous materials protect cells from high external liquid shear (Black *et al.*, 1984). Passive immobilization is generally reversible and therefore weak; washout of adsorbed cells can easily occur if a certain liquid velocity is achieved.

A ceramic support material can be used for the adsorption of cells. The main advantage of such an inert material is that it can be used almost indefinitively, thus reducing the cost of fermentation. Ceramics such as

silica, titania and alumina are chemically inert and stable within a wide range of chemical, temperature and pH-conditions (Rosevear *et al.*, 1987). Their high mechanical strength makes them suitable for continuous fermentation. Non inert materials have also been used successfully for the adsorption of cells. Table 1.6 presents different support materials that have been used recently for immobilizing different cells.

Marcipar *et al.* (1980) tested the degree of affinity of different yeast strains adsorbed onto a ceramic support. The percentage of cells adsorbed was different for every strain of yeast and was generally higher at pH 4 than at pH 6. The results also show that the strength of fixation was a characteristic of the strain and was not dependent on the pH.

Immobilized cells have been used extensively for the rapid production of soy sauce. Porous alumina ceramic beads were used for yeast cell immobilization by Iwasaki *et al.* (1991) and for lactic acid bacteria immobilization by Iwasaki *et al.* (1993). Horitsu *et al.* (1990) used a two-stage bioreactor with cylindrial filamentous ceramics with fixed yeasts (*Zygosaccharomyces rouxii* and *Candida versatilis*) for the production of soy sauce. The fermentation time was 8 days. Horitsu *et al.* (1991) further reduced the fermentation time to 6 days by using ceramic beads for the immobilization of the cells.

Osaki et *al.* (1985), Hamada *et al.* (1989) and Hamada *et al.* (1991) entrapped cells into sodium alginate beads for the rapid production of soy sauce. The total production time required for the sauce was 2 weeks or less.

Objectives

The general objective of the present work was to develop an accelerated process for the production of a canola sauce of comparable quality to commercial soy sauce. Specific objectives were:

- to produce canola sauce with immobilized cells using support materials that have better mechanical and transport properties than alginate beads.

- to produce several batches of canola sauce using the same immobilized cells in order to simulate a continuous use of the biocatalysts.

- to produce traditional canola and soy sauces under the same conditions to investigate if composition of the sauces differs when substituting soybean meal with canola meal.

- to compare the differences in composition between traditional canola sauce and canola sauce produced by the accelerated process with immobilized cells.

Table 1.1 Typical composition of five varieties of soy sauce recognized by the Japanese Government (Fukushima, 1985).

	Koikuchi shoyu	Usukushi shoyu	Tamari shoyu	Saishikomi shoyu	Shiro shoyu
Degree Baumé	22.0	22.2	29.2	26.9	26.9
NaCl[1]	16.9	18.9	19.0	18.6	19.0
Total nitrogen[1]	1.57	1.19	2.55	2.39	0.50
Formol nitrogen[1]	0.94	0.80	1.05	1.11	0.24
Reducing sugar[1]	3.0	4.2	5.3	7.5	20.2
Alcohol[2]	2.3	2.1	0.1	trace	trace
pH	4.7	4.8	4.8	4.8	4.6
Color	deep brown	light brown	dark brown	dark brown	yellow to tan

[1] (%,w/v)
[2] (%,v/v)

Table 1.2 Reactions of koji enzymes (Ningsanond, 1991).

Enzyme	Reaction
Proteinase, serine-type amides	hydrolysis of proteins and peptides
Acid carboxypeptidase	peptidyl-L-amino acid + H_2O = peptide + L-amino acid
Leucine aminopeptidase	aminoacyl-peptide + H_2O = amino acid + peptide
Glutaminase	L-glutamine + H_2O = L-glutamate + NH_3
Alpha-amylase	endohydrolysis of 1,4-alpha-D-glycosidic linkages in polysaccharides containing three or more 1,4-alpha-linked D-glucose units
Glucoamylase	hydrolysis of terminal 1,4-linked alpha-D-glycose residues successively from non-reducing ends of the chains with release of beta-D-glucose
Pectinase	random hydrolysis of 1,4-alpha-D-galactosiduronic linkages in pectate and other galacturonans
Cellulase	endohydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, lichenin and cereal beta-D-glucans

Table 1.3 Sugars, organic acids, and alcohols in koikuchi-shoyu (Fukushima, 1985).

Components	Concentration
Sugars	
Monosaccharide[a]	2.65%
Disaccharide	0.65%
Oligosaccharide	Trace
Polysaccharide	1.15%
Total	4.45% as glucose
Organic acids	
Pyroglutamic acid	0.35%
Lactic acid	0.68%
Acetic acid	0.16%
Formic acid	0.02%
Citric acid	0.04%
Succinic acid	0.05%
Total	1.29%
Alcohols	
Ethanol	2.0-2.5%
n-Butanol	4 ppm
Isobutanol	3 ppm
Isoamylalcohol	5 ppm
2-Phenylethanol	3 ppm
Furfurylalcohol	5 ppm

[a]Mannose, 0.62; arabinose, 0.77; galactose,1.72; xylose, 0.55; and glucose, 20.50 mg/ml.

Table 1.4 Proximate composition and amino acid content of canola meal and soybean meal (Clandinin, 1986).

	Canola Meal	Soybean Meal
Proximate composition (%)[1]		
Moisture	8.01	11.00
Crude Fiber	11.41	7.30
Ether extract	3.35	0.80
Protein (Nx6.25)	37.15	45.21
Amino acid composition[2] (in % of total protein)		
Alanine	4.65	4.31
Arginine	6.08	6.71
Aspartic acid	8.16	11.66
Cystine	2.48	1.61
Glutamic acid	17.38	18.65
Glycine	5.18	4.55
Histidine	2.79	2.48
Isoleucine	3.44	4.03
Leucine	6.92	7.44
Lysine	5.95	6.24
Methionine	2.10	1.59
Phenylalanine	3.90	4.72
Proline	6.67	5.03
Serine	4.55	5.27
Threonine	4.52	3.85
Tryptophan	1.19	1.15
Tyrosine	2.62	2.95
Valine	4.42	4.18

1: These numbers do not add up to 100%, as other compounds such as sugars and ashes are not reported.

2: These numbers do not add up to 100%, as only the major amino acids are reported.

Table 1.5 Immobilization methods for whole microbial cells (Witter, 1992).

BINDING
Weak Bonds
Flocculation
Adsorption
Ionic
Strong bonds
Covalent
Cross-linking
PHYSICAL RETENTION
Entrapment
Thermal gelation
Ionotropic gelation
Polymerization
Membrane retention
Dialysis culture
Ultrafilters

Table 1.6 Various support materials for adsorption of cells.

Support material	Organism	Reference
Polyurethane foam	*Aspergillus niger* *S. diastaticus* *Zymomonas mobilis*	Lee *et al.*, 1989 Amin *et al.*, 1985 Amin *et al.*, 1987
Polystyrene macroporous beads	Mammalian cells	Lee *et al.*, 1992
POLYHIPE™ (porous polystyrene-based polymer)	*Citrobacter freundii*	Griffiths and Bosley, 1993
Lignocellulosic material (bagasse, sawdust, rice-straw, rice-husk)	*S.cerevisiae*	Das *et al.*, 1993
Porous stainless steel	*L. mesenteroides*	El-Sayed *et al.*, 1992
Beech wood chips	*S. cerevisiae*	Moo-Young *et al.*, 1980
Ultraporous fired bricks	Yeast cells	Opara and Mann, 1988
Cotton thread	Yeast cells	D'Souza, 1990
Biofix (derived from kaolinite)	*S. cerevisiae*	Salter *et al.*, 1990
Celite (diatomaceous earth)	*S. cerevisiae*	Nguyen and Shieh, 1992

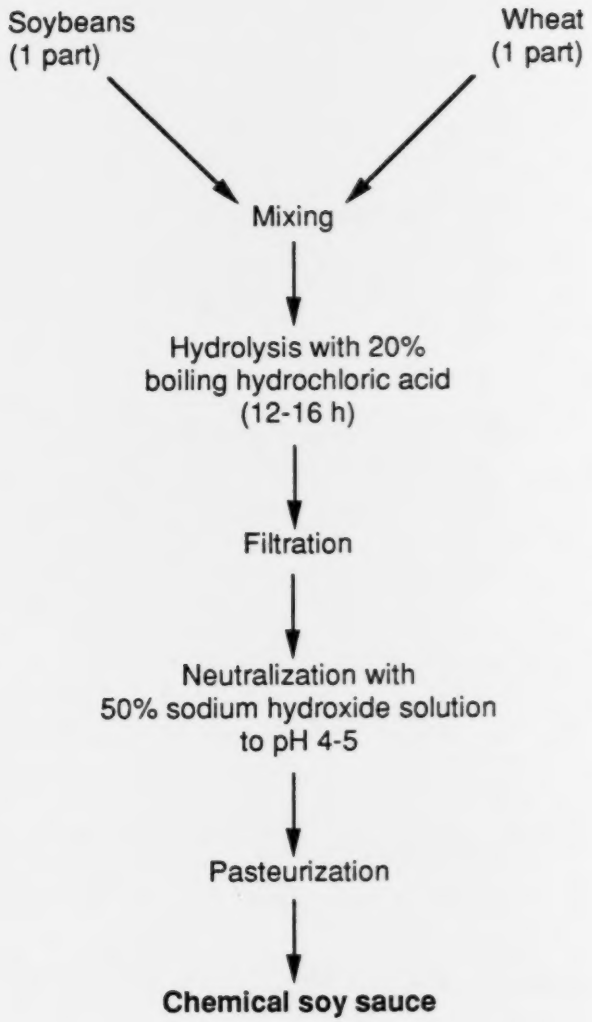

Figure 1.1 Production process of chemical soy sauce (Yong and Wood, 1974).

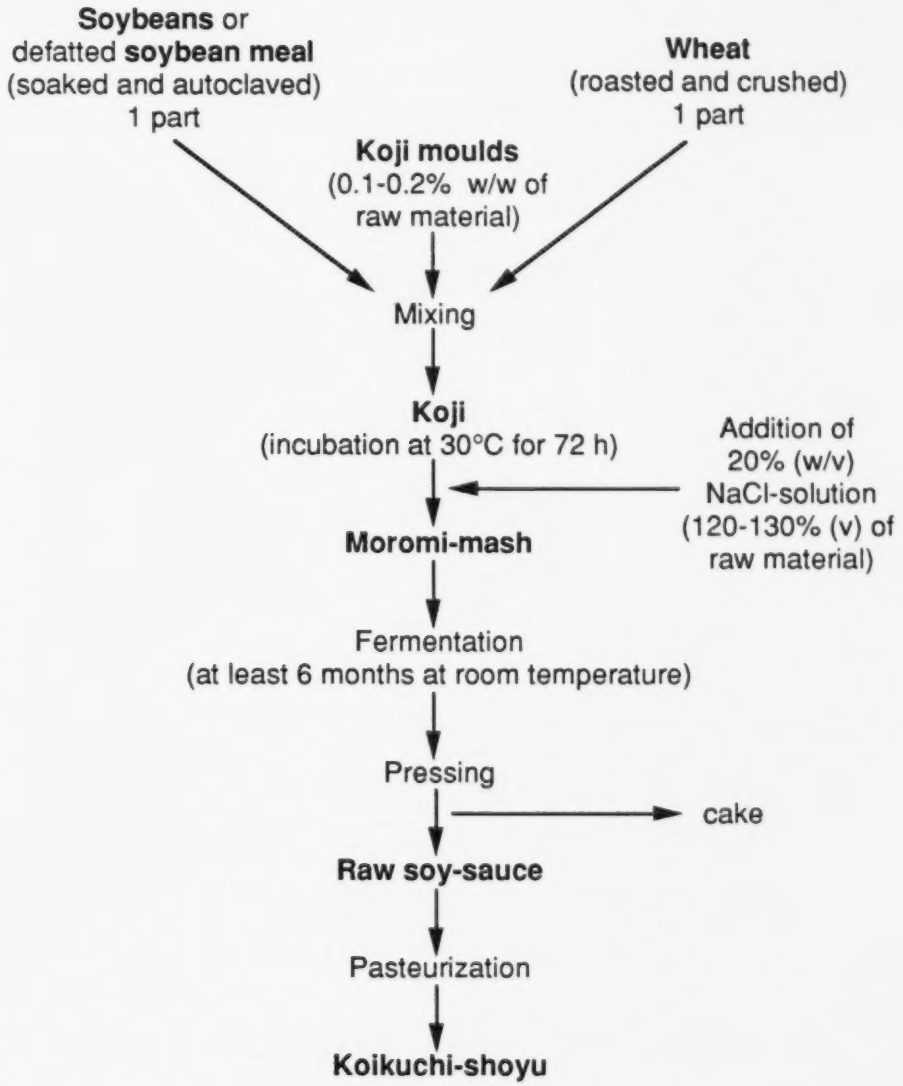

Figure 1.2 Manufacturing process of koikuchi-shoyu (after Fukushima, 1985).

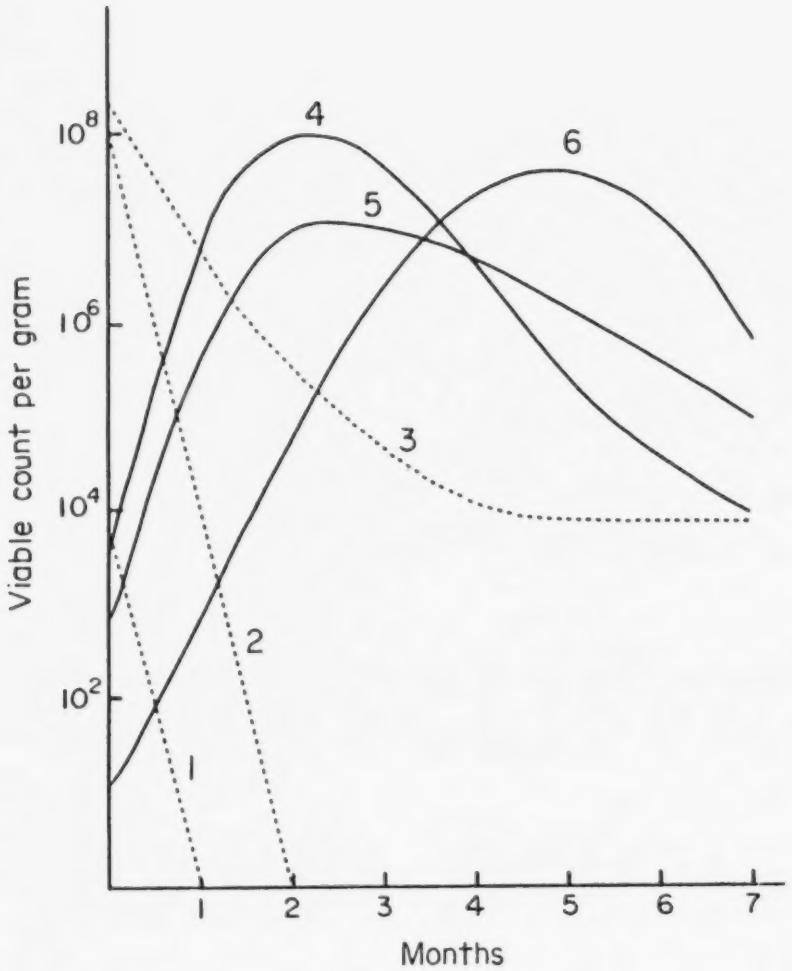

Figure 1.3 Microflora changes in shoyu mash fermentation: 1 wild yeast; 2 *Micrococcus* ; 3 *Bacillus* ; 4 *Lactobacilli* ; 5 *Saccharomyces rouxii* ; 6 *Torulopsis* yeasts (Tamagawa *et al.*, 1975).

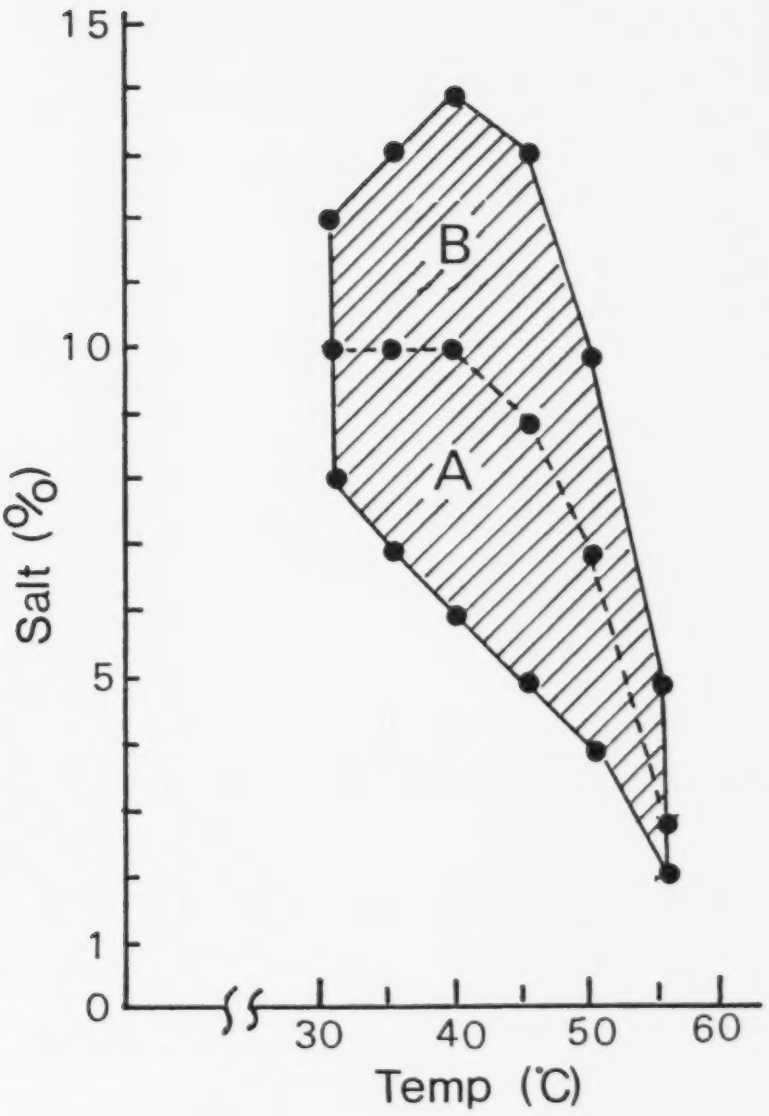

Figure 1.4 Safety zone for enzymatic digestion of shoyu koji (Takamatsu *et al.*, 1980).

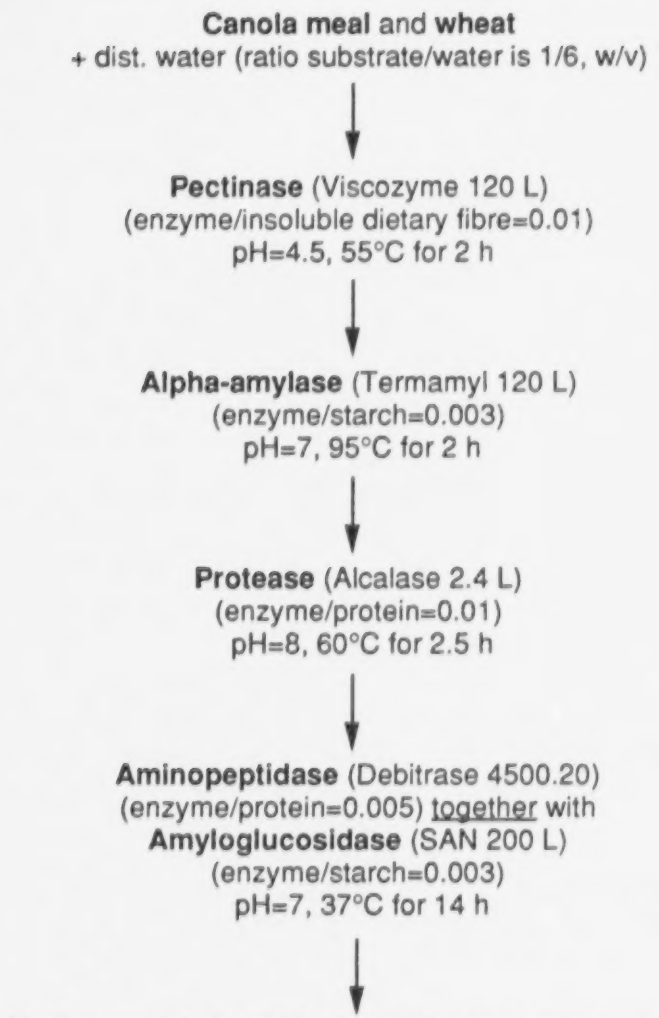
Canola meal and **wheat**
+ dist. water (ratio substrate/water is 1/6, w/v)
Pectinase (Viscozyme 120 L)
(enzyme/insoluble dietary fibre=0.01)
pH=4.5, 55°C for 2 h
Alpha-amylase (Termamyl 120 L)
(enzyme/starch=0.003)
pH=7, 95°C for 2 h
Protease (Alcalase 2.4 L)
(enzyme/protein=0.01)
pH=8, 60°C for 2.5 h
Aminopeptidase (Debitrase 4500.20)
(enzyme/protein=0.005) together with
Amyloglucosidase (SAN 200 L)
(enzyme/starch=0.003)
pH=7, 37°C for 14 h
Hydrolysate (40% TSS, ca. 0.7% (w/v) lactic acid after
centrifugation and concentration)

Figure 1.5 Multi-step hydrolysis of canola meal and wheat using industrial enzymes (after Ningsanond, 1991).

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CHAPTER 2

CANOLA SAUCE PRODUCTION USING IMMOBILIZED YEAST CELLS IN REPEATED BATCH FERMENTATIONS

INTRODUCTION

The traditional production of soy sauce is a two-stage process, consisting of a 'koji' step, where mould grows for 72 h on a mixture of wheat and soybean meal, followed by a 'moromi' fermentation involving koji enzymes, lactic acid bacteria and yeasts. The moromi fermentation, where organic acids and ethanol are produced, takes at least 6 months.

It has been shown that an acceptable soy-like canola sauce could be produced using canola meal instead of soybean-meal (Ooraikul *et al.*, 1980). The production process of the canola sauce has since then been shortened considerably from about one year to several days (Ma and Ooraikul, 1986; Coleman and Ooraikul, 1989 and Ningsanond, 1991).

Using immobilized cells for a fermentation process is an attractive method due to the reuseability of the cells and the possibility of making the system continuous. Entrapping cells in alginate beads is probably the most widely used method for immobilizing cells. This immobilization method was used by Ningsanond (1991) to produce canola sauce and by Osaki *et al.* (1985) for the production of soy sauce. But alginate beads present several problems, such as restricted transport of products through the polymer

(Adams *et al.*, 1988). Moreover, due to their lack of mechanical strength, they are not suitable for long term continuous processes.

Other supports have been used for fermentation processes, which are made of inert materials. Iwasaki *et al.* (1993) immobilized lactic acid bacteria in porous alumina beads for organic acid production in soy sauce. Adams *et al.* (1988) used porous particles derived from the clay mineral kaolinite, commercialized under the name Biofix. These are formed into hollow microspheres in the form of "bird nests" with porous walls. The mechanism of cell immobilization into Biofix has been explained by Salter *et al.* (1990). The cells are immobilized into particles whose mesopore is facing the direction of fluid flow by simple filtration. For particles whose orifice is not facing the direction of flow, the cells enter the particles by a mechanism called hydrodynamic deposition or "lobster-pot" effect. Because the ceramic particles offer a resistance to the flow of the fluid, there will be a pressure drop across them. This will have the effect of forcing cells into the orifice.

The problem with inert materials is their relatively high price. Less expensive support materials, such as polyurethane foam cubes (Amin *et al.*, 1987) and macroporous polystyrene (Lee *et al.*, 1992) have also been used successfully for cell immobilization and need to be tested for the long term use for the production of canola sauce.

This chapter investigates the alcohol fermentation for production of canola sauce using yeast cells immobilized in 2 different support materials: Biofix and Labpor, a porous, high density polyethylene foam.

The cell load after immobilization in the two support particles was compared. Ethanol concentration in the feed medium was monitored during repeated batch processes using the same immobilized yeast cells.

In this study, the usual lactic acid fermentation prior to ethanol fermentation was not required. The amount of organic acids present in the feed medium, especially lactic acid, was already higher than necessary. This was probably due to the presence of an unnaturally high amount of lactic acid bacteria in the building where this study was carried out, as a result of other concurrent studies of these bacteria in an adjacent laboratory, which may have contaminated the koji mixture during the preparation step and mould growth.

MATERIALS AND METHODS

Materials

Defatted canola meal was obtained from CanAmera Foods, Fort Saskatchewan, AB. The meal was ground and sifted through a 40-mesh sieve. Wheat grains were obtained from the Alberta Wheat Pool, Fort Saskatchewan, AB. They were first roasted in a pan until turning slightly brown, then ground and sifted through a 40-mesh sieve.

The porous polyethylene support material Labpor X-6810 (pore size 60 μm), with hydrophilic properties, was obtained from Porex Technologies, Fairburn, GA. The material comes in form of tubes (OD = 6.22-6.60 mm; ID = 3.18-3.38 mm), which were cut, using a surgical knife, in rings with a

thickness of ca. 0.8 mm. The rings could be autoclaved since the softening point of this material is 130°C and the melting point is 160°C.

The ceramic support material Biofix C2, which comes in the form of hollow porous microspheres derived from kaolinite, was obtained from ECC International, Atlanta, GA. The particle size was 50-70 μm, the average cavity entrance and mean wall pore size were 20 μm and 0.5 μm respectively.

Kikkoman soy sauce produced by Kikkoman Foods Inc., Walworth, WI, USA was bought in a local store and used as the reference sample.

Microorganisms and cultivation

Aspergillus oryzae ATCC 14895, *Aspergillus sojae* ATCC 20387 and *Zygosaccharomyces rouxii* (previous name: *Saccharomyces rouxii*) ATCC 13356 were obtained from American Type Culture Collection (ATCC), Rockville, MD.

A. oryzae and *A. sojae* were aerobically cultured on malt extract agar plates, medium 325 (Blakeslee formula) in the ATCC catalogue, composition: 20.0 g malt extract, 20.0 g glucose, 1.0 g peptone, 20.0 g agar, 1.0 L distilled water.

Z. rouxii was cultivated aerobically in shake flask culture (150 rpm) for 72 h at 28°C in YM broth (Difco Laboratories, Detroit, MN.), with addition of 8.5% (w/v) NaCl.

Preparation of canola feed medium for alcohol fermentation

To obtain a solid-free feed medium, canola meal and wheat were hydrolyzed with the enzymes from koji prior to alcohol fermentation.

Koji preparation: 40 g canola meal were soaked in 60.2 mL distilled water for about 24 h and then autoclaved at 121°C for 20 min. After cooling down, 40 g wheat were added. This mixture had a moisture content of about 45%. It was then transfered into a plastic tray and inoculated with about 0.15% (w/w, dry basis) of a mixture of *A. oryzae* and *A. sojae,* and incubated at 28°C for 72 h in a chamber at near saturation humidity. The koji was stirred 20 h and 24 h after inoculation.

Hydrolysis: The mature (72 h old) koji was hydrolyzed using a method adapted from Takamatsu *et al.* (1980). The koji was mixed with a 3% (w/v) NaCl solution and transfered to an Erlenmeyer flask. The amount of solution added was just enough to suspend the mash when the flask was shaken at 200 rotations per minute. This mixture was shaken for hydrolysis at 55°C for 72 h. After hydrolysis, the liquid mash was centrifuged for 20 min. at 9,000 x g and the supernatant was then boiled for 10 min. After cooling down, the feed medium was filtered through No. 4 Whatman filter paper, concentrated in a vacuum evaporator at 40°C from 15% total soluble solids (TSS) to 40% TSS, and finally pasteurized for 30 min. at 80°C. At this stage, the medium had a pH of 4.4 and a lactic acid content of 1.89% (w/v). It was therefore not necessary to carry out a lactic acid fermentation prior to the alcohol fermentation.

Alcohol fermentation

The canola feed medium was subjected to alcohol fermentation at room temperature (22°C) by *Z. rouxii.* The yeast cells were either

immobilized in Labpor or Biofix. For one batch, 25 g (dry weight) of either Labpor or Biofix were used with 150 mL of canola feed medium.

The fermentation with Labpor was carried out in an autoclaved, round bottom glass jar fermenter (capacity 600 mL). Mixing of the particles was achieved by a magnetic stirrer. The fermentation with Biofix C2 was carried out in an autoclavable stirred cell (capacity 350 mL; Model 8400, Amicon, Danvers, MA), where stirring occurs from the top.

When the alcohol content reached about 1.5% (w/v), the medium was drained from the support particles using an autoclaved strainer and immediately replaced by fresh feed medium. The same Biofix or Labpor particles containing yeast cells were used for three consecutive batches.

Refining of the sauce

After about 1.5% ethanol was reached in the sauce and removal of the supported cells, the salt level was adjusted to 18% (w/v) by addition of NaCl. The sauce was then heated to 80°C and held for 20 minutes. After cooling it was centrifuged at 9,000 x g for 20 minutes, filtered through Whatman No.1 paper and finally bottled. A flow chart of the complete production process is shown in Fig. 2.1.

Immobilization of microbial cells

Immobilization of cells into Labpor:

10.1 g wet weight of *Z. rouxii* were suspended in 140 mL 0.2 M phosphate buffer (61 parts by volume of 0.2 M K_2HPO_4 and 39 parts by volume of 0.2 M KH_2PO_4) and 25 g of Labpor rings were added to the cell suspension. After stirring the mixture for 24 h, the cell suspension was

drained aseptically from the rings. The rings were then rinsed with feed medium and transferred to the fermenter.

Immobilization of cells into Biofix:

A column (i.d. 2.8 cm) with a fritted glass disc (pore size: 30-40 µm) at the lower end was packed with 25 g Biofix. 600 mL of a 16.8 g/l (wet weight) suspension of *Z. rouxii* was passed through the Biofix at a velocity of approximately 0.8 cm/s by drawing vacuum from the bottom of the column. To avoid the formation of a yeast skin which could block the column, the top third of the Biofix column was stirred constantly with a sterile spatula, while the cell suspension was flushed through. The cell suspension was collected in a flask and passed through the column again in the same manner. The column was then flushed 4 times with 100 mL distilled water and the Biofix particles were transferred to the fermenter (Biofix was washed with water instead of feed medium because the latter was to viscous to be flushed through the column).

Artificial feed medium for alcohol fermentation

YM broth (Difco Laboratories, Detroit, MN.) was used at a concentration of 10.5 g/L with addition of 5.0% (w/v) glucose and 8.5% (w/v) NaCl. This medium will be referred to as the 'artificial' feed medium.

Scanning electron microscopy

After immobilization of yeast cells, the support materials Biofix and Labpor were air-dried and mounted on double sided sticky tape (Shinto Chemtron STR) on aluminium stubs (Cambridge). They were then sputter

coated with 150 Å of gold in a sputter coater (Nanotec Semprep II) and examined using a scanning electron microscope (Cambridge S 250).

Determination of cell load in the support material after immobilization

After alcohol fermentation, 25 g Labpor or Biofix particles were rinsed very gently 4 times in 500 mL distilled water. They were then boiled in 0.2 M NaOH for 1 h to lyse the cells. The NaOH solution was then assayed for protein by the Folin method (Lowry *et al.*, 1951). The amount of protein was determined with a calibration curve made with Bovine Serum Albumine (BSA). A second calibration curve was made by determining the protein content in known amounts of wet cells. The load of the support was expressed in g cells (wet weight)/kg support material.

Total soluble solids (TSS) measurement

TSS of the refined sauces were measured with an Abbe refractometer (Carl Zeiss, Germany).

pH measurement

pH was measured with a Fisher Accumet Selective Ion Analyzer Model 750 (Fisher Scientific Co., Pittsburgh, PN).

Amino-nitrogen (AN)

Free amino-nitrogen of the samples was determined using method 10.179 from A.O.A.C. (1980). Ninhydrin colour reagent reacts with the

amino-groups of the diluted sample. The amount of amino nitrogen was then determined from the absorbance at 570 nm.

Total soluble nitrogen (TSN)

TSN of the samples was measured using the Kjeldahl method 2.055 from A.O.A.C. (1980).

Analysis of amino acids

The free amino acid profile of the samples was determined using a reverse phase high pressure liquid chromatography (HPLC) method from Jones and Gilligan (1983).

Chromatography system:

Separation and quantification of amino acids was accomplished with the use of a Varian 5000 high performance liquid chromatograph and a Varian Fluorichrom detector (excitation 340 nm, emission 450 nm). Samples were mixed 1:1 with the fluoraldehyde reagent prior to injection. 25 µL of mixed sample were injected onto a Supelcosil 3 micron LC-18 reverse phase column (4.6 x 150 mm; Supelco) equipped with a guard column (4.6 x 50 mm) packed with Supelco LC-18 reverse phase packing (20-40 µm). Chromatographic peaks were recorded and integrated using a Shimadzu Ezchrom Chromatography Data System.

Buffer solutions:

Solvent A (0.1 M sodium acetate) was prepared by adding 11.5 mL glacial acetic acid and 8 g of sodium hydroxide to 1600 mL deionized water.

The pH was adjusted to 7.2, and 180 mL methanol and 10 mL tetrahydrofuran were added, and the volume was adjusted to 2 L with deionized water. Solvent B was methanol.

Solvent A and solvent B were used as the gradient mobile phase, with % solvent B increasing from 0 to 100% over a 30 minute period.

Preparation of fluoraldehyde reagent:

0.5 g of OPA was dissolved in 12.5 mL methanol. 112 mL 0.04 M sodium borate buffer (pH 9.5) was added along with 0.5 mL 2-mercaptoethanol and 4 mL Brij 35.

Preparation of samples:

To 0.1 mL sample, 0.1 mL ethanolamine/ß-amino-butyric acid (25 nmol/mL), 0.1 mL saturated $K_2B_4O_7$, and 1.1 mL of water were added.

Analysis of total titratable acidity

The total titratable acidity was determined according to Onaga *et al.* (1957). The method consisted in titrating the diluted sauce sample with NaOH to pH 8. Results were expressed as milliequivalent of acid per 100 mL sauce.

Analysis of ethanol and glycerol

Ethanol and glycerol were determined using the UV-method from the Methods of Biochemical Analysis and Food Analysis (Boehringer Mannheim, 1989). The principle of ethanol determination consisted in producing NADH in an enzymatically catalyzed reaction. The amount of

NADH, which was half stoichiometric with the amount of ethanol present, was measured at 340 nm. The determination of the amount of glycerol involved a reaction that consumed NADH. The amount of NADH oxidized was stoichiometric with the amount of glycerol. The change of NADH was determined from its absorbance at 340 nm.

Analysis of organic acids and sugars

HPLC was used to identify and quantify organic acids and sugars. The system consisted of the following Shimadzu components: SCL-6A system controller, LC-6A solvent delivery unit, SIL-6A auto injector unit, CTO-6A column oven, and C-R6A and C-R3A data processors (Shimadzu Corp., Kyoto, Japan). Column effluents were monitored by a Toso-Haas TSK-6040 UV-VIS detector (Hadley Tekscience, Oakville, ON) and a Shimadzu RID-6A refractive index detector (Shimadzu Corp., Kyoto, Japan). An Aminex HPX-87H cation exchange column with a cation H^+ Microguard Precolumn (Bio-Rad Laboratories, Mississauga, ON) was used for separations. The UV detector was set at 220 nm and was used to quantify organic acids, while the RI detector was used to quantify sugars. The system was operated at a column temperature of 25°C and a flow rate of 0.5 mL/min for sugars, and at 65°C and a flow rate of 0.7 mL/min for organic acids. 0.009 N H_2SO_4 was used as the mobile phase. The 12 fold diluted samples were filtered through a 0.45 μm Millipore membrane (Millipore Corp., Bedford, MA) and then passed through a solid phase extraction column (SCX, Phenomex, Torrance, CA) and a SEP-PAK C_{18} cartridge (Waters Associates, Inc., Milford, MA) before injection. Injection volume was 20 μL.

Determination of sodium chloride

Sodium chloride in the sauce was determined using a Fisher Accumet Selective Ion Analyzer Model 750 (Fisher Scientific Co., Pittsburgh, PN), which was attached to an Orion Sodium Ion sensing electrode and a single junction reference electrode (Orion Research Inc., Cambridge, MA). The analyzer was set on the concentration mode and was calibrated with 0.4 and 0.1% NaCl standard solutions adjusted with an ionic strength adjustor (ISA) solution.

Colour measurement

The colour of the sauce samples was measured with a Hunterlab model D25/L-2 colorimeter (Hunter Associates Laboratory Inc., Fairfax, VA).

RESULTS AND DISCUSSION

Alcohol production in repeated batch fermentations

The alcohol content of a fermented sauce is an important quality criterion. It was rated highest as the chemical compound which contributed most to the preference of soy sauce (Yokotsuka, 1972). Values of 2-2.5% for a soy sauce are reported in literature (Fukushima, 1985). High ethanol content means also longer fermentation time, therefore it is important to reach a compromise between product quality and production time. For this study, it was decided to stop a fermentation at alcohol content of 1.5% (w/v), which is still in the range of a typically 'fully brewed' sauce (Wood, 1982).

Fig. 2.2 (solid lines) and 2.3 show the ethanol concentration in moromi broth with respect to time in three consecutive batches using the same yeast cells. The organisms were immobilized in either Biofix or Labpor. The average fermentation time to reach 1.5% alcohol was 120 h (5.0 d) for Biofix, whereas for Labpor, it took 174.5 h (7.3 d).

When the cells were immobilized in Biofix, two batches could be performed with the same cells. For the third batch, the alcohol production slowed down considerably and stopped after reaching about 1%. When the cells were immobilized in Labpor, only one satisfactory batch could be performed. For the second batch, the productivity slowed down and during the third batch no alcohol was produced. In each case it could be observed that the alcohol productivity of the cells slowed down considerably or stopped after about 300 h. These results indicate that the cells probably died or were somehow inhibited. Fouling of the support could also be the cause of alcohol production loss. Fouling is the deposition of suspended materials around or in the pores of the support. It can prevent access of substrate to the cell causing a loss of activity. Fouling is enhanced by viscous, concentrated substrates (Cheetham, 1985). In our case this could have been one reason for activity loss as the feed medium contains 39.5% TSS.

Although lactic acid bacteria have not been added to the canola feed medium, it contained already 0.7% lactic acid after the hydrolysis step and 1.89% after concentration to 40% TSS. The koji pH of 4.4 after hydrolysis was already in the optimum range to start an alcohol fermentation. Usually the pH of fresh moromi mash ranges from 6.5-7.0, depending upon the pH

value of the koji, which can be affected by contamination with acid-forming bacteria such as *Micrococcus* (Yokotsuka, 1985).

Fig. 2.4 shows the lactic acid content in the hydrolyzate during hydrolysis of the mature koji. Most of the lactic acid is formed during the first 24 h of hydrolysis. This high amount of lactic acid might be due to the unusually high occurrence of lactic acid bacteria in the atmosphere of the building where this study was carried out. As the koji incubation is not carried out under sterile conditions, lactic acid bacteria might have contaminated it during this time. To confirm this hypothesis, two batches of koji followed by enzymatic hydrolysis were prepared in a different building where presence of lactic acid bacteria, other than the naturally occuring, was very unlikely. The resulting feed medium after concentration to 40% TSS only contained 0.01% (w/v) lactic acid.

Although a lactic acid content of almost 2% is too high for a medium undergoing yeast fermentation, as it might slow down or inhibit cell growth and alcohol production, it is important to realize that the total moromi fermentation time can be reduced if lactic acid bacteria are inoculated before the koji making or the hydrolysis step. The total production cost would be reduced since the formation of lactic acid and other organic acids can be carried out simultaneously with the koji step and the hydrolysis of the raw material. The inoculation should be done under controlled conditions in order to get an amount of organic acids similar to that of a commercial sauce. It is also possible to use other lactic acid forming bacteria, since the specific property of the high salt tolerance of *P. halophilus* is not needed with only 3% (w/v) sodium chloride in the hydrolyzate, compared to 18% for the traditional moromi fermentation. But it would be necessary to investigate

which effect the presence of organic acids might have on the action of koji enzymes.

To see whether the high amount of lactic acid in the moromi broth had an influence on the alcohol productivity of the yeast cells, the feed medium with a lactic acid content of 0.01 %, was brought to a lactic acid content of 0.7%, which is close to the value of Kikkoman sauce, by mixing it with medium containing 1.89% (w/v) lactic acid. It was then subjected to an ethanol fermentation using *Z. rouxii* cells immobilized into Biofix. The broken line in Fig. 2.2 shows one batch of alcohol fermentation using the medium containing 0.7 % (w/v) lactic acid. The total time to obtain 1.6% alcohol is only 67.5 h as compared to 120 h. These results suggest that the activity of the cells is slowed down if the alcohol fermentation is carried out with a medium containing as high as 1.89% lactic acid as in the case described previously. Due to a limited amount of medium containing 0.7% lactic acid, only two separate batches could be performed, but it would be of interest to know how many consecutive batches can be performed using the same cells, and also if the alcohol production will slow down as observed when using the high lactic acid feed medium.

It also needs to be investigated if the cells can be revitalized after the alcohol production slows down. The support particles with immobilized cells could be transfered to a growth medium and then be reused for a batch of alcohol production. The possible fouling of the support particles also needs to be investigated.

In order to determine the possible reasons for the loss of activity of the yeast cells, similar experiments were carried out using the 'artificial' feed medium with *Z. rouxii* immobilized in Labpor. It was observed that the cells

could be used at least 5 times without any noticeable loss of activity or contamination problems, as shown in Fig. 2.5. The time required to produce 1.5% alcohol was 76.1 h (3.2 days) on average. Therefore it is probable that the feed medium used for canola sauce fermentation reduced the activity of the cells and/or caused fouling of the support material.

Choice of support material and immobilization method

Fig. 2.6 and 2.7 show electron microscopy photographs of *Z. rouxii* immobilized in Biofix and Labpor respectively. When examining Labpor particles under the electron microscope, it was observed that the cells were mainly located just under the surface of the particles. Few cells were found deep inside the plastic. There seems to be a repulsion between this material and the yeast cells.

Before adopting the immobilization method of simply stirring the yeast suspension and Labpor particles, we first tried to immobilize the cells into the pores under reduced pressure. A flask containing Labpor particles was set under vacuum (25 inches of mercury) and a yeast cell suspension was run into the flask without releasing the vacuum. The vacuum was then released, which should have forced the suspended cells into the pores of the support. However, the amount of immobilized cells did not differ between the two methods.

When immobilizing Biofix, the method with the glass column and vacuum used in this study was found to be easier to handle and to scale up than the method using a syringe proposed by Salter *et al.* (1990).

The amount of cells immobilized in Biofix was higher than those in Labpor, which explains the higher productivity. The average cell load was

27.1(±2.4) g cells (wet weight)/kg support material and 15.6(±2.2) g/kg support material for Biofix and Labpor, respectively.

In our experiments the cell loading of Biofix was slightly lower than those achieved by Salter *et al.* (1990), which were 25-40 g wet yeast/kg of Biofix. One reason for the reduced cell load could be that the opening of the Biofix particles was smaller than indicated by the manufacturer. By examining the particles under the electron microscope, it could be seen that only few particles had an opening with diameter close to 20 μm. Most had openings between 3 and 15 μm. If during immobilization the yeast cells were in clusters, only few cells would have a chance to penetrate the ceramic particles. It was also observed that the liquid velocity of 0.8 cm/s through the column during immobilization could only be achieved at the beginning of the immobilization step. As the suspension was forced through the column, the fritted glass probably became blocked with smaller Biofix particles.

The advantage of this immobilization method into Biofix is that the cells do not need to be harvested from the growth medium prior to immobilization, as they can be immobilized directly with the growth medium. In this work, however, the cells were first harvested and then suspended in phosphate buffer. Furthermore, the cell load in the particles could probably be increased if after immobilization the cells are allowed to grow inside the particles. However, there might be a problem to supply sufficient oxygen for cell growth, as the growth medium might foam under intense aeration conditions.

Biofix has several advantages over Labpor: it could be used almost indefinetively, as it is resistant to chemical attack, whether by chelating

agents, acids or alkalis; it is thermally stable to 1000°C and can be autoclaved without loss of surface area or porosity. Labpor is made of high density polyethylene and therefore is not as resistant. It could be autoclaved a few times in our experiments, but would probably deteriorate with further autoclaving.

Choice of bioreactor

Because the feed medium in this study was very viscous, a packed bed was not a good choice because of possible clogging and channeling problems. A stirred tank reactor became the reactor of choice for this study. The support particles were mixed through stirring.

An air lift reactor initially seemed to be a good choice. However, foaming problems could not be overcome even when using antifoaming agents and mechanical foam breakers. Fermentation is often accompanied by foam formation because of gas production and high foaming capacity of protein solutions. This capacity results from stabilization of the gas/liquid interface. Under extreme conditions it is possible that the entire medium will be converted into foam and, consequently, will be lost (Schuegerl, 1985).

The stirred tank reactors used for these two support materials proved to be suitable. Since the density of Biofix ceramic particles is higher than that of the feed medium, a simple stirring bar could damage the particles by grinding the material, as it is not very resistant to abrasion. By using an Amicon stirring cell, this problem was solved, as the stirring occurs from the top (impeller), resulting in no damage to the ceramic particles.

The fact that Biofix comes in form of a powder could make its handling on a larger scale difficult (e.g. dust formation, recovery of particles without loss after fermentation).

The density of Labpor particles was lower than that of the bulk liquid and therefore they floated on the surface of the feed medium. By using a fermenter with a stirring bar at the bottom, the particles were distributed uniformly throughout the liquid. However a higher stirring speed was required for the Labpor particles than for the Biofix particles. A problem with intense stirring is that cells might be dislodged from the support material due to the high shear stress near the stirring bar.

Composition of the sauce

Table 2.1 to 2.6 show the composition of the sauce made using yeast cells immobilized in Biofix and Labpor and the feed medium before fermentation. The differences in composition between canola sauce and commercial soy sauce will be described in Chapter 3. The general composition of the canola sauce compares well with the commercial Kikkoman sauce even though a deliberate lactic acid fermentation was not done for the canola sauce.

The amount of lactic acid and other organic acids is generally higher in our canola sauce, probably due to contamination with lactic acid bacteria during the koji step. The strain *Z. rouxii* used in this study has been found to be inhibited by acetic acid (Noda et al., 1982). This could have slowed down the alcohol production process for the canola sauce. Therefore, another strain of *Z. rouxii* with less sensitivity to acetic acid should be tested in future reactions.

It can be noticed that part of the amino nitrogen, organic acids and sugars are consumed by the yeast cells during the fermentation process, as indicated by the generally higher amount of amino nitrogen and organic acids in the feed medium before fermentation compared to canola sauce.

CONCLUSIONS

Repeated batch fermentations were possible using immobilized *Z. rouxii* cells, although in this study cell activity was reduced after the first or second batch, probably due to high amounts of organic acids in the feed medium and/or fouling of the support material. With artificial feed medium, however, at least 5 consecutive batches were possible using the same immobilized yeast cells, without any noticeable loss of activity.

Biofix was found to be a better support material than Labpor. The cell load in Biofix was higher and the support could be used almost indefinitively.

Table 2.1 Total soluble solids (TSS), NaCl-content, ethanol and glycerol content of canola and commercial Kikkoman sauces (average of duplicate determinations on all batches).

	CF[a]	CS	KS
TSS (%)	39.5±0.0	38.0±0.7	35.5±0.0
NaCl (% w/v)	9.35±0.10	18.0±0.5	14.8±0.7
Ethanol (% w/v)	0.00	1.51±0.06	1.67±0.06
Glycerol (% w/v)	0.00	0.362±0.014	1.84±0.06

[a]CF: canola feed medium before ethanol fermentation (single sample was analyzed).
CS: canola sauce produced with yeast cells immobilized on Labpor and Biofix C2 (6 batches were analyzed).
KS: Kikkoman soy sauce produced by Kikkoman Foods, Inc. (single sample was analyzed).

Table 2.2 Total soluble nitrogen (TSN), amino nitrogen (AN) and ratio AN/TSN of canola and commercial Kikkoman sauces (average of duplicate determinations on all batches).

	CF[a]	CS	KS
TSN (% w/v)	2.29±0.02	1.79±0.09	1.50±0.01
AN (% w/v)	0.990±0.006	0.775±0.033	0.729±0.018
AN/TSN	0.49	0.43	0.49

[a]CF: canola feed medium before ethanol fermentation (single sample was analyzed).
CS: canola sauce produced with yeast cells immobilized on Labpor and Biofix C2 (6 batches were analyzed).
KS: Kikkoman soy sauce produced by Kikkoman Foods, Inc. (single sample was analyzed).

Table 2.3 Total acidity (meq NaOH/100 mL sauce), pH and organic acid content (mg/100 mL sauce) of canola and Kikkoman sauces (average of duplicate determinations on all batches).

	CF[a]	CS	KS
Total acidity	30.2±0.0	25.2±1.4	18.8±0.0
pH	4.40±0.01	4.52±0.07	4.77±0.0
Organic acids			
Lactic	1892.4±10.7	1591.8±68.3	628.1±4.2
Pyroglutamic	173.0±0.9	148.6±5.8	373.0±0.6
Acetic	353.1±0.8	255.9±11.7	94.7±0.4
Formic	97.4±0.2	91.8±3.6	77.0±0.4
Citric	471.2±0.9	388.8±12.2	145.4±0.9
Propionic	538.2±6.5	573.4±5.6	217.3±4.8
Succinic	505.8±2.8	273.1±5.9	82.4±0.9
Malic	U/O[b]	U/O	U/O
Pyruvic	U/O[c]	U/O	U/O

[a]CF: canola feed medium before ethanol fermentation (single sample was analyzed).
CS: canola sauce produced with yeast cells immobilized on Labpor and Biofix C2 (6 batches were analyzed).
KS: Kikkoman soy sauce produced by Kikkoman Foods, Inc. (single sample was analyzed).
[b]: unable to estimate due to overlap with pyruvic acid.
[c]: unable to estimate due to overlap with malic acid.

Table 2.4 Amino acid profile of canola and Kikkoman sauces (μmol/mL, average of duplicate determinations on all batches).

Amino acid	CF[a]	CS	KS
GLU	68.1±1.8	61.5±3.4	52.6±0.7
ASP	23.7±0.4	21.2±2.2	8.14±0.04
SER	14.8±0.6	11.6±1.0	31.1±0.5
HIS	9.36±0.13	7.58±0.4	7.84±0.07
GLY	47.0±1.7	32.5±3.6	25.0±0.7
THR	19.2±0.1	15.6±1.5	17.8±0.1
CIT	1.57±0.03	1.17±0.09	2.43±0.04
ARG	3.08±0.14	2.43±0.41	20.3±0.7
TAU	10.6±0.4	6.79±0.86	0.00
ALA	42.8±0.5	37.2±2.6	55.3±0.8
TYR	9.47±1.01	7.79±0.90	2.71±0.04
TRP	4.31±0.05	2.89±0.26	0.415±0.014
MET	6.09±0.22	4.87±0.96	4.29±0.01
VAL	25.5±0.3	22.9±2.1	25.0±0.1
PHE	16.0±0.3	13.0±1.6	16.8±0.2
ISO	19.8±0.3	18.1±1.2	22.0±0.1
LEU	41.2±0.94	34.3±1.7	35.1±0.3
ORN	17.5±0.7	14.8±0.7	0.877±0.074
LYS	16.6±0.5	14.3±1.8	18.5±0.6

[a]CF: canola feed medium before ethanol fermentation (single sample was analyzed).
CS: canola sauce produced with yeast cells immobilized on Labpor and Biofix C2 (6 batches were analyzed).
KS: Kikkoman soy sauce produced by Kikkoman Foods, Inc. (single sample was analyzed).

Table 2.5 Sugar content of canola and Kikkoman sauces (g/L, average of duplicate determinations on all batches).

	CF[a]	CS	KS
Glucose	19.4±0.3	1.13±0.37	4.16±0.12
Arabinose	1.39±0.01	0.983±0.103	0.529±0.027
Sucrose and Maltose[b]	3.54±0.06	3.03±0.25	2.87±0.01
Xylose, Mannose and Galactose[c]	2.65±0.14	2.16±0.20	3.65±0.14

[a]CF: canola feed medium before ethanol fermentation (single sample was analyzed).
CS: canola sauce produced with yeast cells immobilized on Labpor and Biofix C2 (6 batches were analyzed).
KS: Kikkoman soy sauce produced by Kikkoman Foods, Inc. (single sample was analyzed).

[b]: results expressed in g Sucrose + Maltose/L because of their coelution in the chromatogram.

[c]: results expressed in g Xylose + Mannose + Galactose/L because of their coelution in the chromatogram.

Table 2.6 Color of canola and commercial Kikkoman sauces (average of duplicate determinations on all batches).

	CF[a]	CS	KS
L - value[b]	14.9±0.1	16.7±1.0	17.4±0.0
a - value[c]	10.2±0.1	10.7±0.8	13.1±0.0
b - value[d]	3.4±0.0	3.2±0.9	3.5±0.1
Color	dark brown	dark brown	dark brown

[a]CF: canola feed medium before ethanol fermentation (single sample was analyzed).
CS: canola sauce produced with yeast cells immobilized on Labpor and Biofix C2 (6 batches were analyzed).
KS: Kikkoman soy sauce produced by Kikkoman Foods, Inc. (single sample was analyzed).

[b]:0 = black, 100 = perfect white

[c]:+ = red, 0 = gray, - =green

[d]:+ = yellow, 0 = gray, - = blue

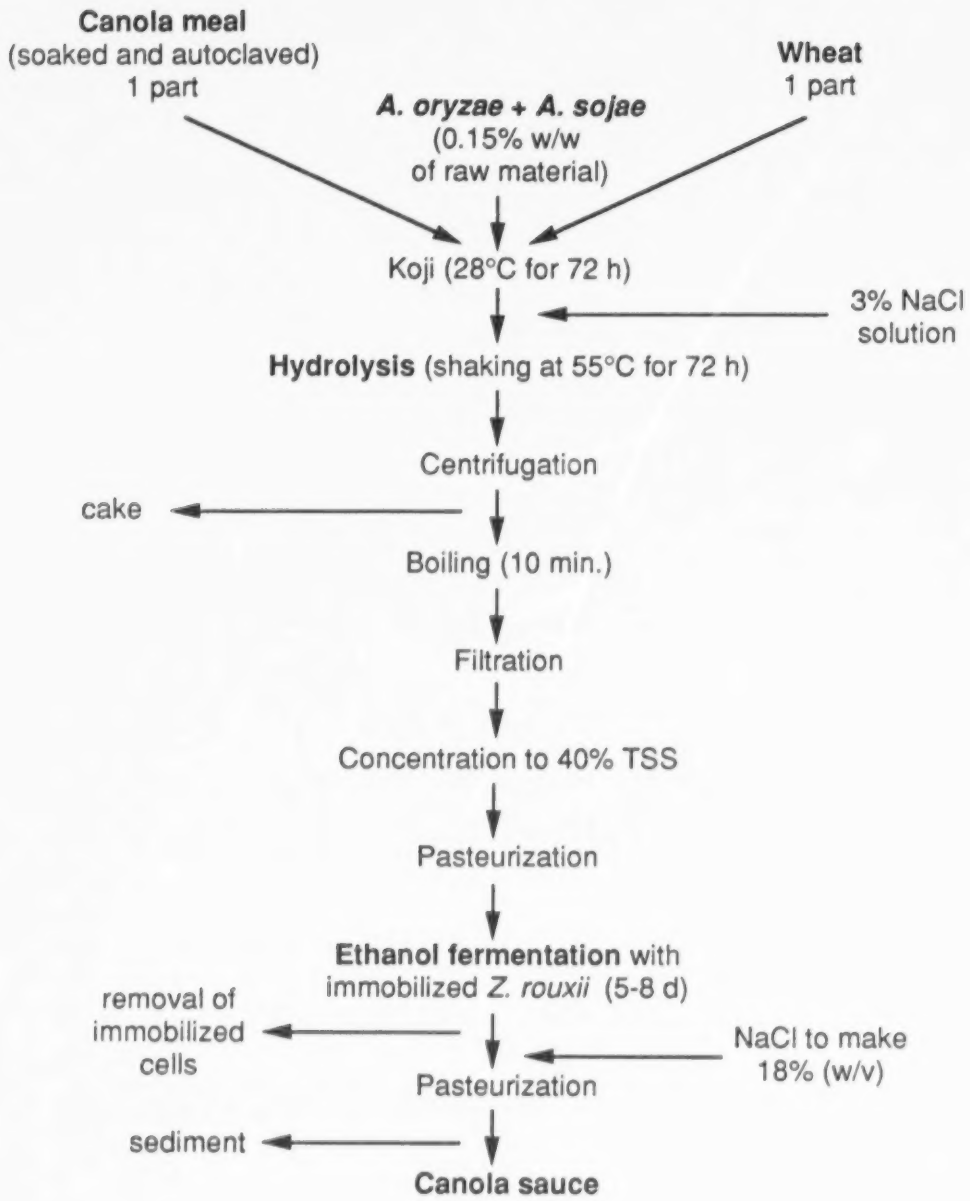


Figure 2.1 Production process for canola sauce.

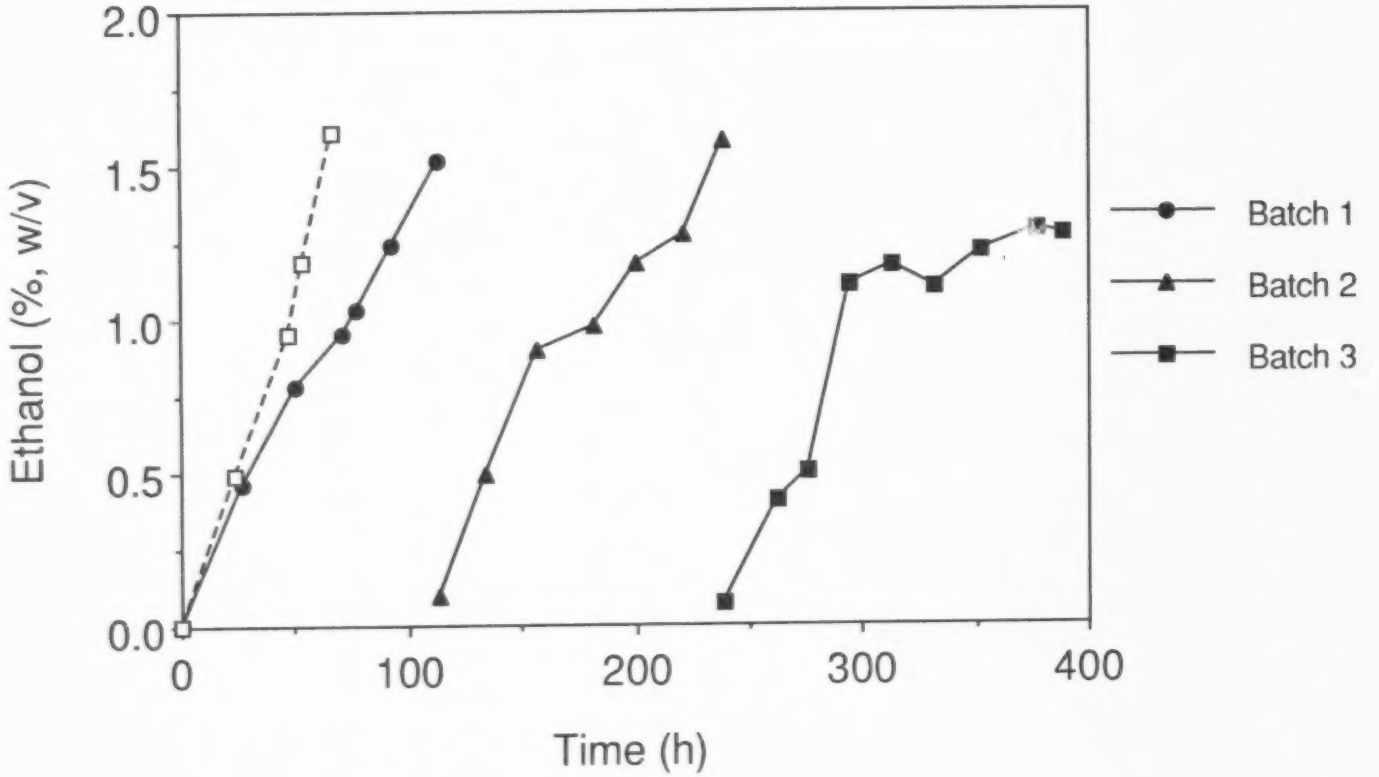


Figure 2.2 Ethanol content in moromi broth during consecutive batches using the same yeast cells immobilized in Biofix C2. (The broken line shows a comparative batch with moromi broth containing 0.7% (w/v) lactic acid).

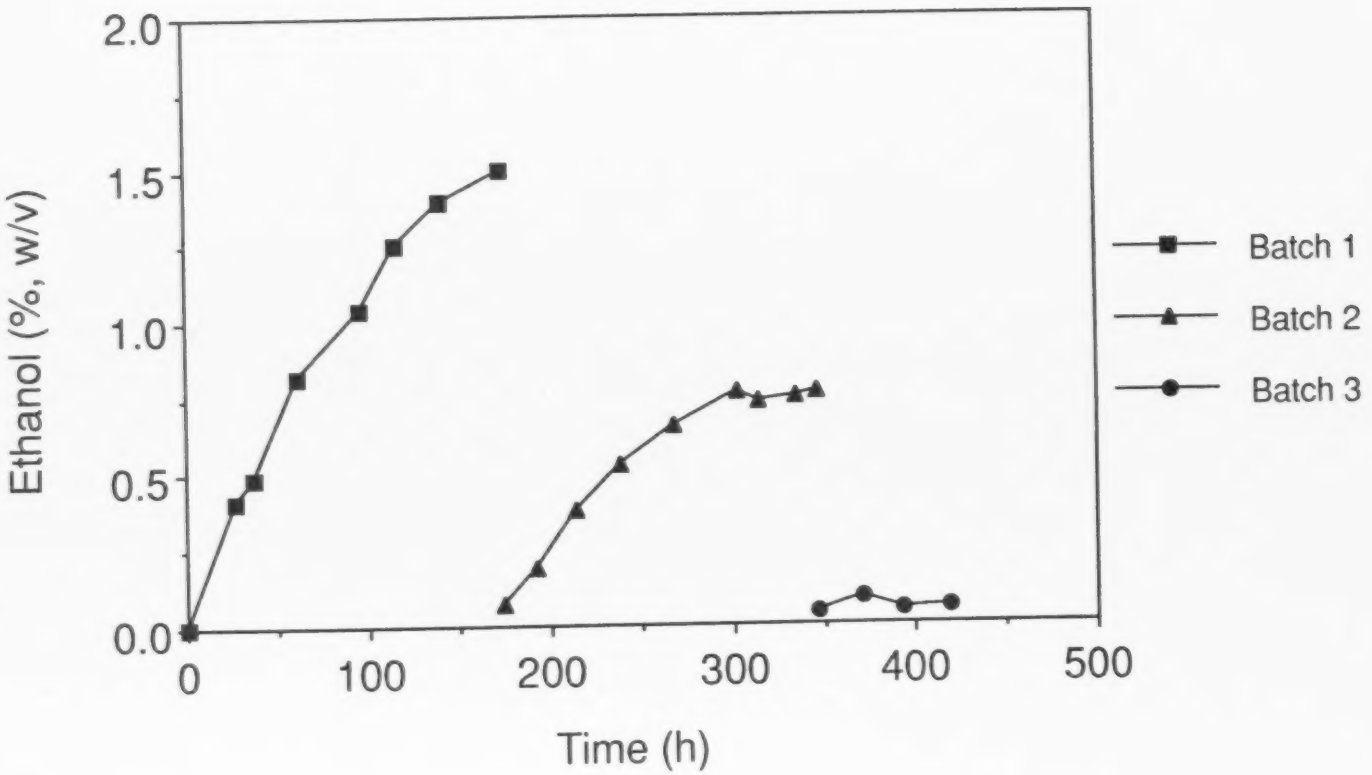


Figure 2.3 Ethanol content in moromi broth during consecutive batches using the same yeast cells immobilized in Labpor.

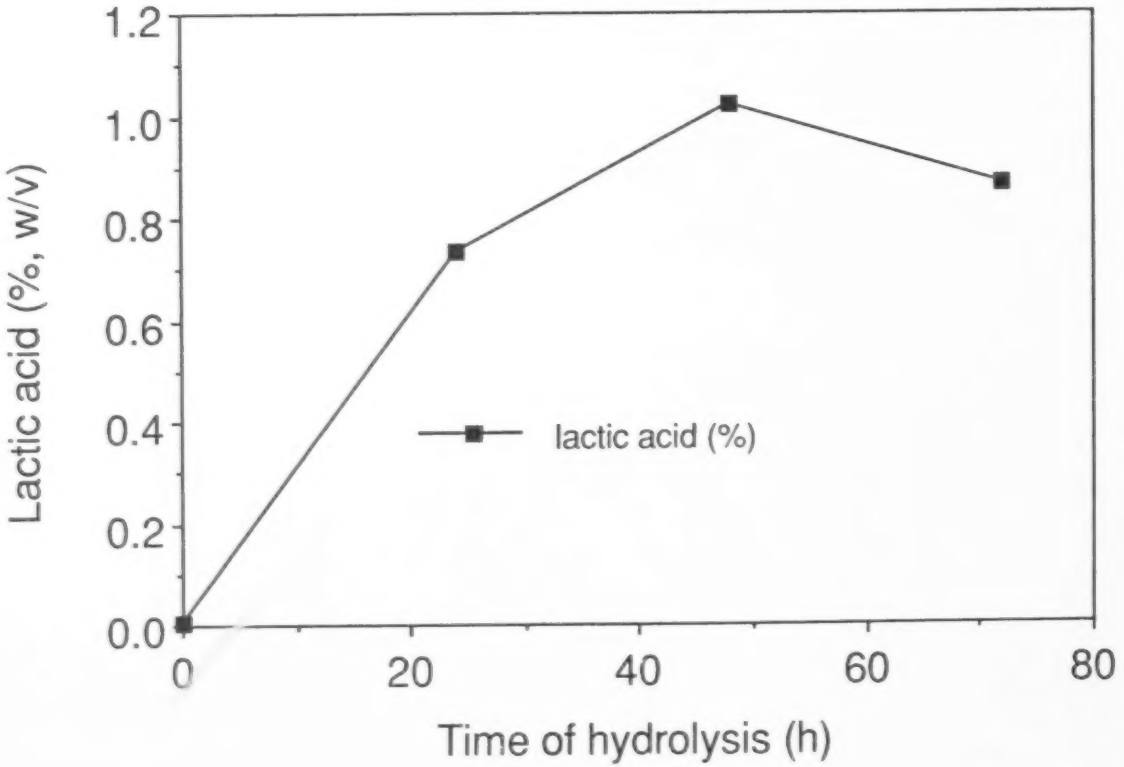


Figure 2.4 Lactic acid concentration during hydrolysis of mature koji.

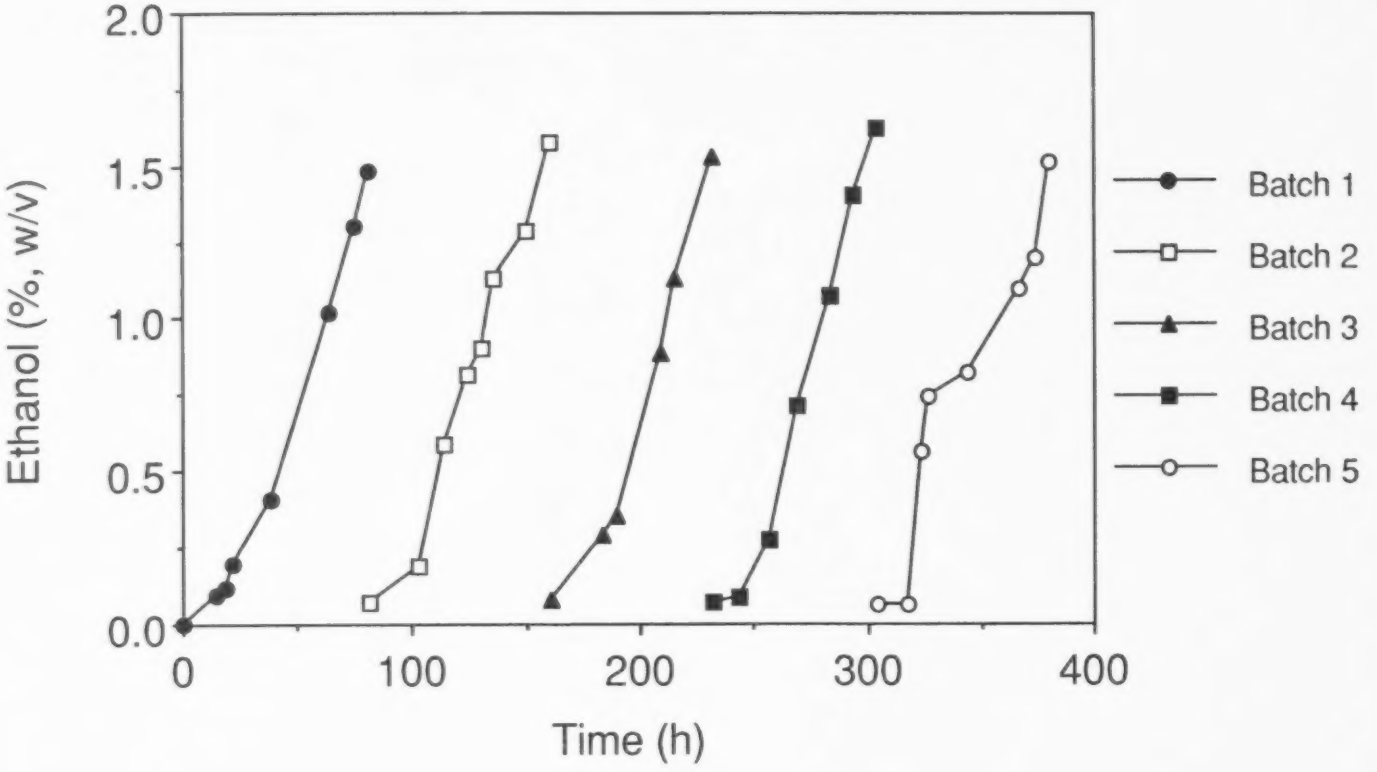
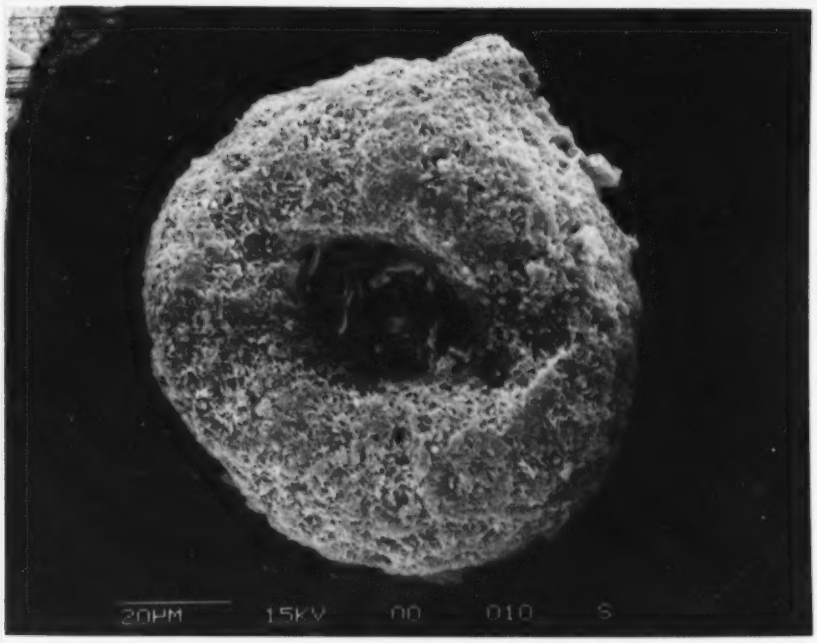


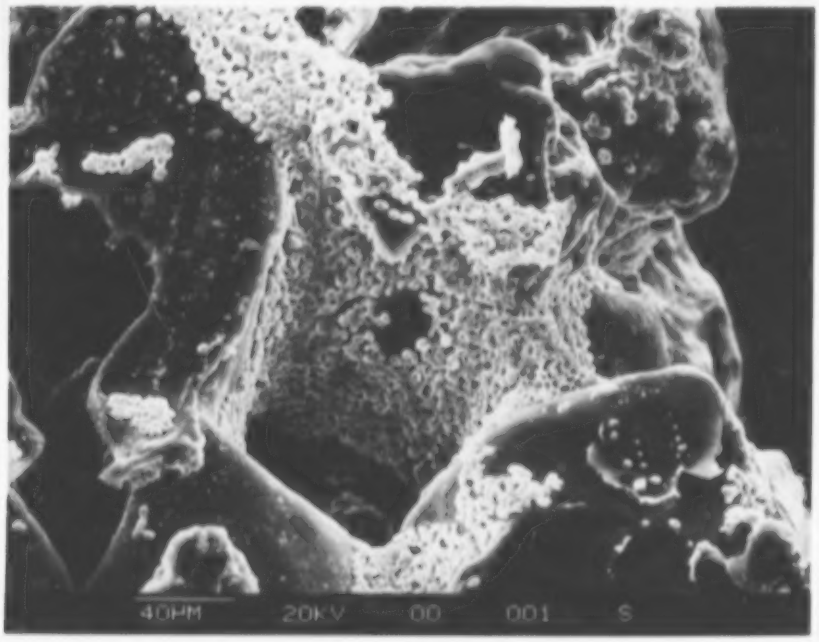
Figure 2.5 Ethanol content in 'artificial' feed medium during consecutive batches using the same yeast cells immobilized in Labpor.



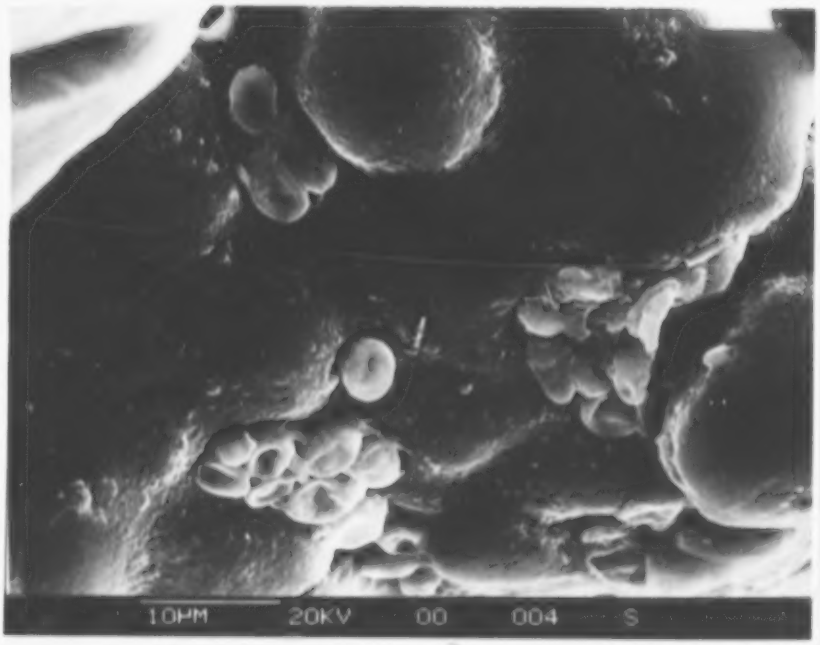
A

B

Figure 2.6 Scanning electron micrographs of *Z. rouxii* cells immobilized inside Biofix C2.
A: Biofix C2 particle
B: Enlargement of the opening of Biofix C2, showing cells inside the 'nest' (bar represents 3 μm; the 0 μm was due to machine display malfunction)



A



B

Figure 2.7 Scanning electron micrographs of *Z. rouxii* cells immobilized in Labpor.
A: outer surface of Labpor
B: inner surface of Labpor

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CHAPTER 3

COMPARISON OF THE COMPOSITION OF TRADITIONAL SOY, TRADITIONAL CANOLA AND 'QUICK' CANOLA SAUCES

INTRODUCTION

Soy sauce is a popular, oriental all-purpose seasoning made from soybeans and wheat. The traditional manufacture is a two-step batch process involving the biochemical action of three types of microorganisms: mould, lactic acid bacteria and yeasts (Noda, 1980). Mould is first grown on a mixture of soybeans and roasted wheat for 3 days. This mass, called koji, is then mixed with a 20% (w/v) sodium chloride solution and then subjected to an anaerobic fermentation for about one year. During this step, also called moromi fermentation, the enzymes from koji break down the raw material into smaller molecules, whereas lactic acid bacteria and yeast cells produce mainly lactic acid and alcohol respectively. The liquid obtained after this fermentation is the typical commercial soy sauce.

It is possible to accelerate the fermentation process by inoculating the moromi mash with pure cultures of lactic acid bacteria and yeast cells (Yokotsuka, 1986b). This, however, requires large scale cultivation of microorganisms each time soy sauce mash is fermented. The repeated fermentation with immobilized cells may be a more effective alternative than

using free cells. To further accelerate the process, it is necessary that lactic acid fermentation and alcohol fermentation be done separately. The yeasts and lactic acid bacteria involved in the fermentation have a complicated interaction with each other, which affects the production of lactic acid and ethanol in each fermentation (Inamori *et al.*, 1984).

Due to the unavailability or high cost of soybeans in certain regions, several attempts have been made to find substitutes for soybeans or soybean meal (Church, 1923 and Tsukahara, 1948). Ooraikul *et al.* (1980) replaced it with canola meal, a by-product of oil extraction process, and produced a canola sauce with characteristics comparable to commercial soy sauce. The total production process has since then been reduced to a few days (Ma and Ooraikul, 1986; Coleman and Ooraikul, 1989 and Ningsanond, 1991).

Ningsanond replaced the traditional koji step by using different industrial enzymes to hydrolyze the wheat and canola meal. The enzymes used by Ningsanond were similar to those found in the traditional koji process: commercial protease, aminopeptidase, alpha-amylase, amyloglucosidase and pectinase. A disadvantage with this process is that each commercial enzyme has a different pH and temperature optimum. After every single enzyme step the temperature had to be adjusted and considerable amounts of NaOH or HCl had to be added to the canola meal/wheat mixture to adjust the pH. Therefore this process is expensive and would also be difficult to scale up. Moreover, the industrial aminopeptidase did not seem to effectively break down the peptide bonds, and therefore the ratio of total soluble nitrogen (TSN) to amino-nitrogen

(AN) in the canola sauce was lower than that of the commercial soy-sauce (Ningsanond, 1991).

An alternative method of hydrolysis of the substrates must be used in order to complete the hydrolysis before the moromi fermentation is initiated if immobilized cells are to be used. Osaki *et al.* (1985) and Hamada *et al.* (1991) completed the hydrolysis of soybean and wheat in 72 h with the enzymes formed during the koji step. Their method was adapted after a Japanese patent (Takamatsu *et al.*, 1980). In the present study, the same method of hydrolysis was applied for the rapid production of fermentable canola moromi broth. This broth was then subjected to alcohol fermentation by immobilized yeast cells as described in Chapter 2. The resulting canola sauce was compared to a canola sauce produced by the traditional method in order to determine differences in composition between the two methods. The sauce produced by the action of immobilized cells will be referred to as the 'quick' sauce.

Commercial Kikkoman soy sauce was used as a reference for the production of the canola sauce by Ma and Ooraikul (1986), Coleman and Ooraikul (1989) and by Ningsanond (1991). It is however not clear if the differences in composition between the commercial sauce and the canola sauce are due to the fact that the sauce is produced with canola meal instead of soybean meal or to the fact that the production process is accelerated. Therefore in this study a soy sauce produced in the same manner as the traditional canola sauce was compared to 'quick' canola sauce as well as to traditional canola sauce. In addition, all the sauces were compared to commercial Kikkoman sauce.

Furthermore, it would be of interest to know which method gives a higher yield in sauce from the same amount of starting raw material, the traditional canola sauce or the 'quick' canola sauce.

This chapter investigates the difference in composition and colour of these different sauces.

MATERIALS AND METHODS

Materials

Defatted canola meal was obtained from CanAmera Foods, Fort Saskatchewan, AB, and defatted soybean meal from CanAmera Foods, Toronto, ON. Both meals were ground and sifted through a 40-mesh sieve. Wheat grains were obtained from the Alberta Wheat Pool, Fort Saskatchewan, AB. They were first roasted in a pan until turning slightly brown, then ground and sifted through a 40-mesh sieve.

Kikkoman soy sauce produced by Kikkoman Foods Inc., Walworth, WI, USA was bought in a local store and used as the reference sample.

Microorganisms and cultivation

Aspergillus oryzae ATCC 14895, *Aspergillus sojae* ATCC 20387, *Zygosaccharomyces rouxii* ATCC 13356 and *Tetragenococcus halophilus* (previous name: *Pediococcus halophilus*) ATCC 21786 were obtained from American Type Culture Collection (ATCC), Rockville, MD.

A. oryzae and *A. sojae* were aerobically cultured on malt extract agar plates, medium 325 (Blakeslee formula) in the ATCC catalogue, composition: 20.0 g malt extract, 20.0 g glucose, 1.0 g peptone, 20.0 g agar, 1.0 L distilled water.

Z. rouxii was cultivated aerobically in shake flask culture (150 rpm) for 72 h at 28°C in YM broth (Difco Laboratories, Detroit, MN.), with addition of 8.5% (w/v) NaCl.

T. halophilus was statically cultured in sodium acetate broth (medium 977 in the ATCC catalogue, composition: 33.0 g sodium acetate, 10.0 g glucose, 3.0 g yeast extract; 10.0 g peptone, 5.0 g K_2HPO_4, 5.0 g NaCl, distilled water up to 1.0 L).

Preparation of traditional soy sauce

Koji preparation: 40 g defatted soybean meal were soaked in 60.2 mL distilled water for about 24 h and then autoclaved at 121°C for 20 min. After cooling to room temperature, 40 g wheat were added. This mixture had a moisture content of about 45%. It was then inoculated with about 0.15% (dry weight basis) of a mixture of *A. oryzae* and *A. sojae* and incubated at 28°C for 72 h in a chamber at near saturation humidity. The koji was stirred 20 h and 24 h after inoculation.

Moromi fermentation: The mature koji (72 h old) was mixed with 140 mL aliquotes of 20% (w/v) NaCl solution and transferred to 500 mL Erlenmeyer flasks. Every flask was then inoculated with approximately 500,000 cells of *Z. rouxii* and *T. halophilus* and held at 27°C with stirring three times weekly. After 4 months, the moromi mash was centrifuged for 20 min. at

9,000 x g to obtain the raw canola sauce, which was then pasteurized for 30 min. at 80°C. After cooling down, the sauce was again centrifuged for 20 min. at 9,000 x g and filtered through No.1 Whatman filter paper.

Preparation of traditional canola sauce

The canola sauce was prepared the same way as the traditional soy sauce, except that canola meal was used instead of defatted soybean meal.

Preparation of 'quick' canola sauce using immobilized yeast cells

Koji was prepared as described under preparation of traditional canola sauce. The mature koji was then hydrolyzed using a method adapted from Takamatsu *et al.* (1980), as follows. The 72 h old koji was mixed with a 3% NaCl solution and transfered to an Erlenmeyer flask. The amount of solution added was just enough to suspend the mash when the flask was shaken at 200 rotations per minute. This mixture was hydrolyzed while shaking at 55°C for 72 h. After hydrolysis, the liquid mash was centrifuged for 20 min. at 9,000 x g and the supernatant was then boiled for 10 minutes. After cooling, the feed medium was filtered through No. 4 Whatman filter paper, concentrated in a vacuum evaporator at 40°C from 15% to 40% total soluble solids (TSS), and finally pasteurized for 30 minutes at 80°C. At this stage, the medium had a pH of 4.4 and a lactic acid content of 1.89% (w/v). It was therefore not necessary to carry out a lactic acid fermentation. The feed medium was then subjected to ethanol fermentation for 5-8 days using immobilized *Z. rouxii* as described in Chapter 2.

The measurement of **total soluble solids** (TSS), **pH** and **color**, as well as the analysis of **amino-nitrogen** (AN), **total soluble nitrogen** (TSN), **total titratable acidity**, **ethanol** and **glycerol**, **organic acids** and **sugars** are described in Chapter 2.

RESULTS AND DISCUSSION

Table 3.1 shows total soluble solids (TSS), salt, ethanol and glycerol content in the different sauces. The ethanol content of all sauces compares favourably with data from literature. Wood (1982) stated that a typically 'fully brewed' soy sauce contains 1-2% (v/v) ethanol. The time required for the 'quick' sauce to reach 1.5% (w/v) was 6 d on average, whereas the traditional sauces were fermented for 4 months to obtain the same amount of ethanol. Ethanol formation is an important step in the process. It is known that the typical aroma of soy sauce does not develop without alcoholic fermentation (Wood, 1982).

The total soluble solids in the sauces ranged from 34 to 38%. The 'quick' sauce had the highest value, but it should be noted that the raw unfermented 'quick' sauce had been concentrated from about 15% TSS to 40% TSS prior to fermentation. In the future, it only needs to be concentrated to 37% or 38% TSS, to be comparable to Kikkoman, which contained 35% TSS. This will result in a higher yield of sauce per kg of raw material. The traditional canola sauce had a slightly lower TSS content than the traditional soy sauce. This might be due to the better digestibility of

soybean meal compared to canola meal, and/or to the higher amount of undigestable fibre in canola meal.

The salt contents of the three sauces prepared in our laboratory were very similar (about 18%). The salt content of the 'quick' sauce was measured after fermentation and then salt was added in order to obtain 18% (w/v). Kikkoman sauce, on the other hand, had a salt content of only 14%.

One advantage of the 'quick' sauce process is that the sauce after fermentation only contains about 10% Sodium chloride, which can then be adjusted to the desired salt level. Low salt sauces are now on the market due to the increased awareness of the negative health effect of high salt consumption (LaBell, 1988).

The glycerol content of all sauces ranged from 0.187 to 1.84%. Kikkoman was the only sauce with values comparable with literature data. This is probably due to the fact that Kikkoman sauce is made with whole soybeans. It has been reported that soy sauce made from whole beans has a higher glycerol content than soy sauce made from defatted beans or meal (Yokotsuka, 1986b). The amount of glycerol in a sauce mainly derived from the degradation of soybean oil is estimated to be about 0.5% (Yokotsuka, 1986b). Glycerol is an important compound of soy sauce, as it contributes to the sweetness. It is detectable when more than 0.5% is present in the sauce (Yokotsuka, 1960). Therefore the glycerol content of the sauces prepared in this study was not adequate, resulting in a lack of the sweetness typical of a good quality sauce.

It was surprising to find that the quick canola sauce, which was fermented with 10% NaCl, contained more glycerol than the canola and soy

sauces produced the traditional way, which contained around 18% sodium chloride. Usually glycerol formation is a response to environmental stress such as high salt concentration (Board, 1983).

Table 3.2 shows the total soluble nitrogen (TSN), amino nitrogen (AN) and the ratio AN/TSN in the sauces. The values for both traditional sauces compare well with Kikkoman sauce. Although the hydrolysis time for the 'quick' sauce was only 72 h, the amino nitrogen content and the soluble nitrogen content of the 'quick' sauce are similar to that of the commercial sauce. The high degree of hydrolysis achieved could be due to the fact that the canola meal and roasted wheat used in this study were sieved through a 40 mesh sieve. This high surface area of the raw material probably permits a greater hydrolytic activity of the koji enzymes. It is also possible that soaking and autoclaving the canola meal prior to koji making favoured the high degree of hydrolysis. This shows an improvement over the method employed by Ningsanond (1991), who used a multi-step hydrolysis with industrial enzymes. It proves that the proteinases and aminopeptidases produced by *A. sojae* and *A. oryzae*, as well as the hydrolysis time of 72 h, were adequate. The traditional soy sauce showed higher values of both AN and TSN than the traditional canola sauce. This is possibly due to the fact that soybean meal has a protein content of 45.21% compared to 37.15% for canola meal.

The quality of soy sauce depends on the ratio AN to TSN. A value of 0.5 or more is considered good (Hesseltine and Wang, 1972). Ma and Ooraikul (1986) and Coleman and Ooraikul (1989) also prepared the koji using the traditional method and obtained satisfactory values for the ratio AN/TSN, whereas Ningsanond (1991), using industrial enzymes, only

obtained an average value of 0.15. This shows that it is difficult to simulate the complex and unique enzyme composition of traditional koji, containing the necessary enzymes in a well-balanced ratio (Fukushima, 1985).

Table 3.3 shows pH, total acidity and organic acid content of the different sauces. The pH of all samples was below 5. The sauces prepared in this study generally had a slightly lower pH than the commercial one. This was probably due to the higher amount of organic acids, the most important being lactic acid.

It was surprising that although the total titratable acidity was higher for the canola sauces and the soy sauce prepared in this study, the pH only differed slightly. This indicates that the hydrolyzate had a very strong buffering capacity.

The content of other organic acids except pyroglutamic acid of the sauces produced in this study was also higher than that of the Kikkoman sauce, possibly due to the action of lactic acid bacteria, which might have contaminated the koji during the incubation. The production process of the 'quick' canola sauce is depicted in Fig. 2.1, Chapter 2. The presence of lactic acid bacteria was also noticed by Ningsanond (1991), who reported 0.7% (w/v) lactic acid in his hydrolyzate after only 14 h of incubation for the enzymatic hydrolysis. This was, however, explained partially by the action of acid forming microorganisms present in the enzyme preparations (Ningsanond, 1991). Coleman and Ooraikul (1989) reported a high content of lactic acid in their samples (up to 1.88 %), giving the sauce a sharp taste. They inoculated the moromi mash with pure cultures of *P. halophilus*, but it was not clear if the high amount of lactic acid came only from the pure cultures or also from other lactic acid bacteria present in the lab

environment. Coleman and Ooraikul's study, as well as Ningsanond's, were carried out in the same building as the present work, where lactic acid bacteria are routinely used in other parts of the building.

Table 3.4 shows the free amino acid profile for the different sauces. The amino acid content of the 'quick' canola sauce is comparable with that of the traditional sauces and Kikkoman sauce, although significant differences exist for some acids.

The most important amino acid in soy sauce is glutamic acid. Yokotsuka (1960) echoed Udo's (1931b) statement, that mainly glutamic acid is responsible for the specific taste of soy sauce. All prepared sauces have sufficient glutamic acid if compared to the commercial sauce. There are significant differences in the content of aspartic acid, serine, arginine, taurine ornithine and tryptophan between the traditional soy and canola sauces. These differences are, however, not observed when comparing soybean meal and canola meal. Furthermore, the content of almost every amino acid is higher in the soy sauce than in the canola sauce. This is consistent with the fact that traditional canola sauce has a lower AN content than traditional soy sauce. The main differences between the 'quick' and traditional canola sauces are in the content of tryptophan, aspartic acid and serine.

The differences between the sauces prepared in this study and the commercial Kikkoman sauce might be due to the fact that Kikkoman Foods Inc. (Walworth, WI) used *A. sojae* as the sole organism for koji, as indicated on the product label, which might result in a different composition of protein hydrolyzing enzymes.

Table 3.5 presents the sugar composition in the different sauces. It was impossible to determine the content of some sugars separately, as sucrose and maltose coeluted in the HPLC chromatogram, as well as xylose, mannose and galactose. As an approximation, the calibration runs for these sugars were done by mixing equal parts (by weight) of the coeluting sugars and expressing the results in g/L maltose and sucrose and in g/L xylose, mannose and galactose.

A good quality soy sauce should contain 2-5% reducing sugar, with glucose accounting for 80% (Fukushima, 1985). Although glucose is the major sugar found in the analyzed sauces, its value ranges only from 0.113 to 0.824% (w/v), which is not adequate for a high quality sauce. Even Kikkoman had only a sugar content of 0.416%. The 'quick' canola sauce had the lowest glucose content with only 0.113% (w/v). It is probable that a substantial amount of glucose and other sugars was consumed by bacteria during the hydrolysis step for the formation of organic acids. This would explain the low sugar content and the high acid contents of the 'quick' sauce. The sugar content before alcohol fermentation was, therefore, just high enough for the alcohol production.

The sugar content of the traditional soy sauce was generally higher than that of the traditional canola sauce. This was surprising, as wheat was the main source for starch degrading enzymes, and both sauces were produced with the same amount of wheat.

The color of the different sauces is presented in table 3.6. Although the fermentation time was very short compared to the traditional process of at least 6 months, the color of the 'quick' sauce compared well with that of the Kikkoman sauce, the latter being slightly more reddish. There was no

significant difference in color between traditional soy and traditional canola sauce.

The dark color of the 'quick' sauce was probably due to the various heat treatments necessary for its production. The mature koji was hydrolyzed for 72 h at 55°C, followed by a boiling step to kill the mould. Later, before the sauce was bottled, there were two pasteurization steps of 20 min each at 80°C. For comparison, the traditional sauces lacked the dark color typical of soy sauce, probably due to inadequate aging of the sauce during the moromi step. There was no significant difference in colour between the traditional soy sauce and the traditional canola sauce.

During the traditional production process of soy sauce, half of the color is produced during the moromi fermentation and aging of the mash, while the other half is produced during the pasteurization step (Yokotsuka, 1986a). This clearly indicates that, if heat treatment is used for the quick sauce, the desirable dark color can be made similar to that of commercial sauce.

Fig. 3.1 compares the yield of canola sauce produced by two different methods from the same amount of starting material. With 40 g canola meal and 40 g wheat, 26 ml of quick sauce (38% TSS) can be obtained, whereas 70 mL sauce (31% TSS) are obtained by using the traditional method. It is obvious that the traditional method is more economical from a raw material utilization point of view. By using the 'quick' hydrolysis, a large amount of undigested material was wasted. The degree of hydrolysis might be improved by prolonging the hydrolysis time of 72 h, but there would be a greater risk of contamination or formation of undesirable compounds.

CONCLUSIONS

The main differences between the 'quick' and traditional canola sauces were the contents of glycerol, some amino acids and color. Traditional soy sauce had higher AN, TSN, and residual sugar contents than traditional canola sauce.

Commercial soy sauce was low in lactic acid and sugar, compared to values found in literature, but the values for the traditional as well as the 'quick' canola sauce compared well with the Kikkoman sauce. Therefore, the 'quick' canola sauce could possibly compete in the North American market, although its low sugar and glycerol content would probably translate in the 'quick' sauce lacking the sweetness typical of a good sauce.

Table 3.1 Total soluble solids (TSS), NaCl-content, ethanol and glycerol content of soy, canola and commercial Kikkoman sauces (average of duplicate determinations on all batches).

	SS[a]	CS1	CS2	KS
TSS (%)	34.0±0.9	31.5±0.6	38.0±0.7	35.5±0.0
NaCl (% w/v)	18.3±0.9	17.6±0.8	18.0±0.5	14.8±0.7
Ethanol (% w/v)	1.73±0.12	1.62±0.08	1.51±0.06	1.67±0.06
Glycerol (% w/v)	0.293±0.016	0.187±0.007	0.362±0.014	1.84±0.06

[a]SS: soy sauce produced the traditional way with 4 month-moromi fermentation (2 batches were analyzed).
CS1: canola sauce produced the traditional way with 4 month-moromi fermentation (2 batches were analyzed).
CS2: 'quick' canola sauce produced with immobilized cells (6 batches were analyzed).
KS: Kikkoman soy sauce produced by Kikkoman Foods, Inc. (single sample was analyzed).

Table 3.2 Total soluble nitrogen (TSN), amino nitrogen (AN) and ratio AN/TSN of soy, canola and commercial Kikkoman sauces (average of duplicate determinations on all batches).

	SS[a]	CS1	CS2	KS
TSN (% w/v)	1.46±0.03	1.20±0.01	1.79±0.09	1.50±0.01
AN (% w/v)	0.681±0.011	0.530±0.009	0.775±0.033	0.729±0.018
AN/TSN	0.47	0.44	0.43	0.49

[a]SS: soy sauce produced the traditional way with 4 month-moromi fermentation (2 batches were analyzed).
CS1: canola sauce produced the traditional way with 4 month-moromi fermentation (2 batches were analyzed).
CS2: 'quick' canola sauce produced with immobilized cells (6 batches were analyzed).
KS: Kikkoman soy sauce produced by Kikkoman Foods, Inc. (single sample was analyzed).

Table 3.3 Total acidity (meq NaOH/100 mL sauce), pH and organic acid content (mg/100 mL sauce) of soy, canola and Kikkoman sauces (average of duplicate determinations on all batches).

	SS[a]	CS1	CS2	KS
Total acidity	19.4±0.1	20.1±0.7	25.2±1.4	18.8±0.0
pH	4.64±0.01	4.61±0.02	4.52±0.07	4.77±0.0
Organic acids				
Lactic	1553.1±64.1	1403.4±50.9	1591.8±68.3	628.1±4.2
Pyroglutamic	93.4±2.4	111.2±2.9	148.6±5.8	373.0±0.6
Acetic	148.4±7.3	181.1±5.6	255.9±11.7	94.7±0.4
Formic	72.2±1.7	64.7±3.7	91.8±3.6	77.0±0.4
Citric	312.3±8.1	307.3±10.2	388.8±12.2	145.4±0.9
Propionic	428.7±10.3	482.2±9.6	573.4±5.6	217.3±4.8
Succinic	108.8±4.1	60.4±3.6	273.1±5.9	82.4±0.9
Malic	U/O[b]	U/O	U/O	U/O
Pyruvic	U/O[c]	U/O	U/O	U/O

[a]SS: soy sauce produced the traditional way with 4 month-moromi fermentation (2 batches were analyzed).
CS1: canola sauce produced the traditional way with 4 month-moromi fermentation (2 batches were analyzed).
CS2: 'quick' canola sauce produced with immobilized cells (6 batches were analyzed).
KS: Kikkoman soy sauce produced by Kikkoman Foods, Inc. (single sample was analyzed).
[b]: unable to estimate due to overlap with pyruvic acid.
[c]: unable to estimate due to overlap with malic acid.

Table 3.4 Amino acid profile of soy, canola and Kikkoman sauces (μmol/mL, average of duplicate determinations on all batches).

Amino acid	SS[a]	CS1	CS2	KS
GLU	63.7±2.2	52.0±1.6	61.5±3.4	52.6±0.7
ASP	35.1±1.4	4.71±0.53	21.2±2.2	8.14±0.04
SER	33.4±0.7	3.58±0.25	11.6±1.0	31.1±0.5
HIS	7.10±0.92	6.12±0.35	7.58±0.4	7.84±0.07
GLY	33.2±0.4	25.4±1.9	32.5±3.6	25.0±0.7
THR	16.4±1.2	13.8±0.6	15.6±1.5	17.8±0.1
CIT	0.600±0.011	0.464±0.137	1.17±0.09	2.43±0.04
ARG	23.3±3.2	2.88±0.17	2.43±0.41	20.3±0.7
TAU	0.356±0.045	5.17±0.46	6.79±0.86	0.00
ALA	39.8±1.3	45.2±1.2	37.2±2.6	55.3±0.8
TYR	3.62±0.38	9.85±0.34	7.79±0.90	2.71±0.04
TRP	0.728±0.112	0.00	2.89±0.26	0.415±0.014
MET	4.59±0.54	3.80±0.24	4.87±0.96	4.29±0.01
VAL	25.7±2.6	18.1±0.7	22.9±2.1	25.0±0.1
PHE	15.3±0.23	9.94±0.28	13.0±1.6	16.8±0.2
ISO	20.5±0.4	13.1±0.5	18.1±1.2	22.0±0.1
LEU	36.3±2.1	23.2±1.0	34.3±1.7	35.1±0.3
ORN	0.00	11.7±0.5	14.8±0.7	0.877±0.074
LYS	20.8±1.3	11.1±0.9	14.3±1.8	18.5±0.6

[a]SS: soy sauce produced the traditional way with 4 month-moromi fermentation (2 batches were analyzed).
CS1: canola sauce produced the traditional way with 4 month-moromi fermentation (2 batches were analyzed).
CS2: 'quick' canola sauce produced with immobilized cells (6 batches were analyzed).
KS: Kikkoman soy sauce produced by Kikkoman Foods, Inc. (single sample was analyzed).

Table 3.5 Sugar content of soy, canola and Kikkoman sauces (g/L, average of duplicate determinations on all batches).

	SS[a]	CS1	CS2	KS
Glucose	8.24±0.93	4.47±0.51	1.13±0.17	4.16±0.12
Arabinose	1.12±0.20	0.856±0.046	0.983±0.103	0.529±0.027
Sucrose and Maltose[b]	4.48±0.33	4.22±0.36	3.03±0.25	2.87±0.01
Xylose, Mannose and Galactose[c]	5.84±0.21	3.91±0.09	2.16±0.20	3.65±0.14

[a]SS: soy sauce produced the traditional way with 4 month-moromi fermentation (2 batches were analyzed).
CS1: canola sauce produced the traditional way with 4 month-moromi fermentation (2 batches were analyzed).
CS2: 'quick' canola sauce produced with immobilized cells (6 batches were analyzed).
KS: Kikkoman soy sauce produced by Kikkoman Foods, Inc. (single sample was analyzed).
[b]: results expressed in g Sucrose + Maltose/L because of their coelution in the chromatogram.
[c]: results expressed in g Xylose + Mannose + Galactose/L because of their coelution in the chromatogram.

Table 3.6 Color of soy, canola and commercial Kikkoman sauces (average of duplicate determinations on all batches).

	SS[a]	CS1	CS2	KS
L - value[b]	39.1±0.6	37.8±0.6	16.7±1.0	17.4±0.0
a - value[c]	20.4±0.5	22.5±0.8	10.7±0.8	13.1±0.0
b - value[d]	20.8±0.4	20.3±0.5	3.2±0.9	3.5±0.1
Color	light brown	light brown	dark brown	dark brown

[a] SS: soy sauce produced the traditional way with 4 month-moromi fermentation (2 batches were analyzed).
CS1: canola sauce produced the traditional way with 4 month-moromi fermentation (2 batches were analyzed).
CS2: 'quick' canola sauce produced with immobilized cells (6 batches were analyzed).
KS: Kikkoman soy sauce produced by Kikkoman Foods, Inc. (single sample was analyzed).

[b] :0 = black, 100 = perfect white

[c] :+ = red, 0 = gray, - =green

[d] :+ = yellow, 0 = gray, - = blue

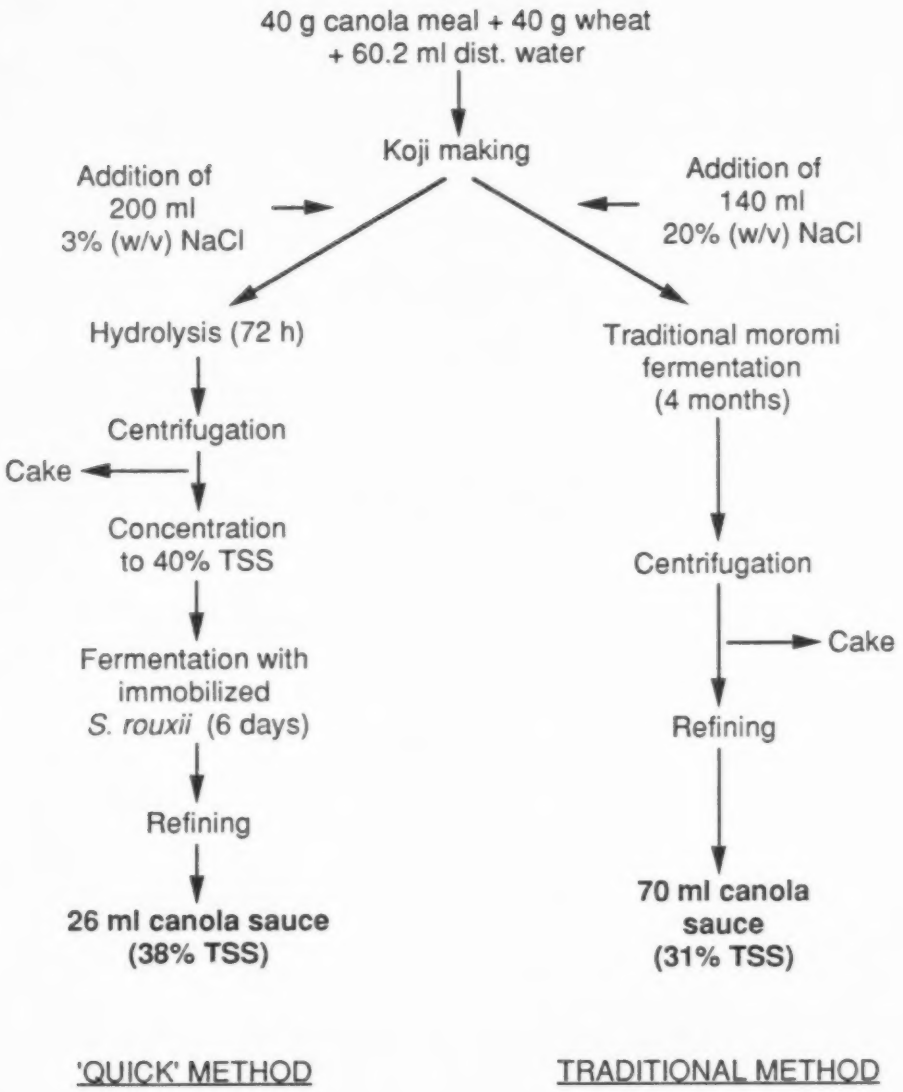

Figure 3.1 Canola sauce yield comparing 2 different production methods.

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CHAPTER 4

GENERAL CONCLUSIONS AND RECOMMENDATIONS

A canola sauce was produced using yeast cells immobilized on two different support materials: Biofix and Labpor. Biofix was found to be a better support with an average cell load of 27.1 g cells (wet weight)/kg support material, whereas only 15.6 g/kg support could be immobilized on Labpor. Consequently, the time to reach the desired alcohol concentration of 1.5% (w/v) was longer with Labpor than with Biofix.

To simulate a continuous use of the cells, consecutive batch runs were carried out using the same supported yeast cells. The activity of the cells was found to decrease after the first or second batch, depending on support material. The time required to obtain 1.5% (w/v) alcohol was between 5 to 8 days. This was considerably longer than the process times reported by Ningsanond (1991) and Hamada *et al.* (1989), where similar alcohol levels were reached in 12 and 28 h respectively. The longer fermentation time was partially explained by the fact that our feed medium contained 1.89% (w/v) lactic acid without ever having undergone a deliberate lactic acid fermentation, probably as a result of contamination of the koji mixture during its preparation and incubation. However, fouling of the support material could also explain the reduced alcohol production rate. When fermentations were performed with a medium containing 0.7% (w/v) lactic acid, the fermentation time was cut in half.

Although 1.89% (w/v) lactic acid content appeared to hinder alcoholic fermentation, the fact that lactic acid and other organic acids necessary for a good sauce can be produced together with the hydrolysis of the raw material was an important finding. By allowing lactic acid production during the koji stage, the sauce production process can be simplified and shortened. The raw material could be inoculated simultaneously with *Aspergillus* cultures and a controlled number of lactic acid bacteria cells in order to obtain about 0.7% lactic acid in the feed medium prior to the alcohol fermentation, thus avoiding the need for a separate lactic acid fermentation stage.

An important consideration in the fermentation with immobilized cells is that the medium must be solid-free. Thus, the hydrolysis of canola meal and wheat has to be completed before the moromi fermentation. In this study, the production of solid free hydrolyzate for alcohol fermentation was prepared after the method of Takamatsu *et al.* (1980), which consisted in using the enzymes from mature koji. This accelerated process represented a good compromise between processing time and sauce quality. An AN/TSN ratio of 0.43 was achieved, which is close to the ideal value of 0.5.

Comparison of the canola sauces produced by immobilized cells and traditional methods revealed that the former had higher glycerol content, but lower sugar content. The canola sauce produced with immobilized cells contains significantly less sugar and glycerol, but twice as much lactic acid than the commercial Kikkoman sauce. In order to be commercially viable, the sweetness of this canola sauce will have to be adjusted.

When traditional soy and canola sauces were compared, it was found that the former had slightly lower TSS, AN, TSN and residual sugar contents, but the overall composition of both sauces compared favourably.

This suggests that canola meal is an acceptable substitute for soybeans or soybean meal.

This study shows that repeated batch fermentation of canola sauce using the same supported yeast cells is possible and can result in an acceptable sauce. However, more research is needed in this area. Future research should include:

1. Production of koji under controlled conditions in which the koji is inoculated with lactic acid bacteria to obtain about 0.7% (w/v) lactic acid in the sauce.

2. Developing a more effective immobilization method for Biofix in order to increase cell load, as well as investigating other support materials.

3. Carrying out several consecutive immobilized cell batch fermentations with the low lactic acid feed medium to simulate continuous operation and determine the useful life of immobilized cells, with and without reactivation between stages.

4. Studying the degree and mechanism of fouling in support media to determine its effect on the activity of the immobilized cells and possible ways to prevent it. Scanning electron microscopy may be useful in studying fouling.

5. Modeling the process taking into account reaction and mass transfer kinetics as well as the effect of various processing parameters.

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